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13. ABSTRACT (Maximum 200 Words)

Our program objective is to develop simple and rapid methods for detecting, at a cellular level, individual responses to environmental stresses elaborated by exposure to infectious agents such as bacteria and viruses. Our methods are based on transcript profiling and post-translational modification of proteins involved in signal transduction. Our hypothesis is that human cells respond to infectious insults to a genetically predetermined extent by stimulating the expression of sets of genes and activating signaling pathways that provide a specific signature for a given agent. We propose that this response will determine the outcome of the infection. We will test this hypothesis by developing custom cDNA and protein arrays designed to detect cellular responses to infectious agents. These will be tested using RNA and protein isolated from tissues sources most likely to be exposed. Our methods will allow development of rapid quantitative detection devices to measure exposure and response to biological warfare, bioterrorism or emerging agents enabling appropriate triaging and medical intervention to save lives and to avoid unnecessary treatments.

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Introduction

There been recent recognition of an increased risk posed by the use of weapons of biological warfare and bioterrorism. By enhancing our ability to respond by rapidly identifying exposed populations and through the triaging of high risk individuals we can overcome both deliberate and naturally occurring pathogen releases. Restricting distribution to exposed and susceptible individuals can also eleviate the shortfall in therapeutic products (when these exist). The rapid testing methods we propose here have as their longterm objective, the detection and identification of individuals who have been exposed to any one of a number of biological agents and at the most risk for succumbing from this exposure. We hypothesize that specific gene and protein expression exist for viruses and bacteria. To test this we have been establishing the gene expression and protein signatures of different important viruses and bacteria that serve as surrogates for highly pathogenic agents. For viruses in which cell culture models exist, RNA is isolated from virus and bacteria-infected cells. In addition, we have been obtaining RNA from infected primary human cells and mouse models. RNA isolated from mammalian cells is converted to dye-labeled complementary DNA or RNA and the labeled nucleic acids used to probe DNA microarrays representing selected human genes implicated in host responses to infection. Viral and bacterial specific signature profiles thus are obtained. Once specific signatures have been established that can discriminate amongst infectious agents we propose construct a composite virus and bacteria induced gene chip as a component of this project. The proposed work will result in technology capable of determining if individuals have been exposed to a life-threatening virus or bacteria and will identify the particular infectious agent by virtue of the signature transcripts induced in the cells. Signal transduction pathways activated by bacterial infection of human cells are being identified and single chain antibodies against a specific signaling component generated.

Body (Progress Report)

Technical Objectives

Assembly and testing of prototype sentinel cDNA and protein microarrays for detecting signatures of biological agents

A. Generating viral specific gene expression profiles from select groups of virological and bacterial agents involved in bioterrorism

Because of restrictions placed on access to and transport of select groups of agents we have continued our work on surrogate agents at our institution as outlined in last year's report. Our focus has been on viruses that infect the respiratory tract. The viruses selected include vaccinia, a surrogate for small pox, and influenza and parainfluenza, because of their potential both as a bioterrorism agents and public health importance. These agents are approved for use in our BSL certified laboratories and can be modeled in mice. Importantly, vaccination programs with vaccinia were implemented and offered the opportunity to obtain specimens from vaccinated individuals that were used to test our arrays and expand the numbers of genes identified as potential sentinels.

1) *in vivo* profiling of response to vaccinia infection.

Vaccinia as a vaccine agent provides approximately 95% cross-reactive protection against clinical disease from variola (smallpox). Yet, as a live virus vaccine, vaccinia can multiply in susceptible hosts, resulting in clinical disease or rarely, death. Vaccinia retains its significant efficacy in preventing smallpox even when given 3-4 days post-exposure (Breman and, Henderson, 2002). Unfortunately, vaccinia is also the most toxic vaccine routinely used during the last century with significant side effects that are local (98%), systemic (20-30%), and rarely lethal (1/million). (Bartlett, 2003). The rate of known side effects is among normal hosts. Particular concern exists with effective immunosuppressive treatments, and solid organ and bone marrow transplantation which results in a larger cohort of persons at increased risk for frequency and severity of side effects. Some areas of cryptic immunodeficiency may also be identifiable through screening. While certain individuals may progress to severe disease because of HIV status (approximately one-third of the estimated 900,000 people in the United States with HIV infection are unaware of their status) immunosuppressive medication or pregnancy, more specific reasons on why certain individuals progress to severe disease are unknown. Of additional serious concern is that twelve of the sixty-eight deaths reported the 1960s occurred in unvaccinated persons exposed to recently vaccinated friends or family members. (Sepkowitz, 2003). Since our work on mice identified a number of vaccinia–induced genes that could serve as sentinels of infection we took advantage of a vaccination program in the health care setting established nationwide as part of the biodefense efforts to measure responses of healthy volunteers using our interferon, dsRNA and stress activated human gene array. As described in last year's report this microarray (termed IAD) comprises approximately 850 known human interferon stimulated genes (ISGs), 1500 human genes containing adenylate-uridylate-rich elements (AREs) and 300 human genes responsive to treatment with the viral analogue dsRNA.

Participants were in one of three groups: A: Pre and 24 hour post testing (n=8); B: Pre and 24 hour testing, with additional samples at 2, 5, 7, and 14 days post vaccination (n=4); or C: Testing at the time of a serious adverse event after vaccination (n=1, generalized vaccinia). Participants served as their own control, except for Group C where enrollment occurred only after onset of the adverse event. Isolated and amplified whole blood RNA was subjected to cDNA microarray testing.

The comparison of pre-vaccination vs 1 day post-vaccination samples revealed that the transcriptional response was highly variable. 71 genes exhibited min 1.8 fold up regulation at 24hrs post-vaccination but these varied among individuals (Figure). We conclude from these preliminary studies that immune activation can be detected in whole blood RNA following live orthopox virus infection using gene expression arrays and that these early immune signatures for orthopox virus infection may assist with identification, containment and treatment decisions. Importantly, significant differences in gene expression profiles were noted in an individual suffering an adverse event (Figure 1) indicating the utility of the arrays in following the outcome of vaccination.

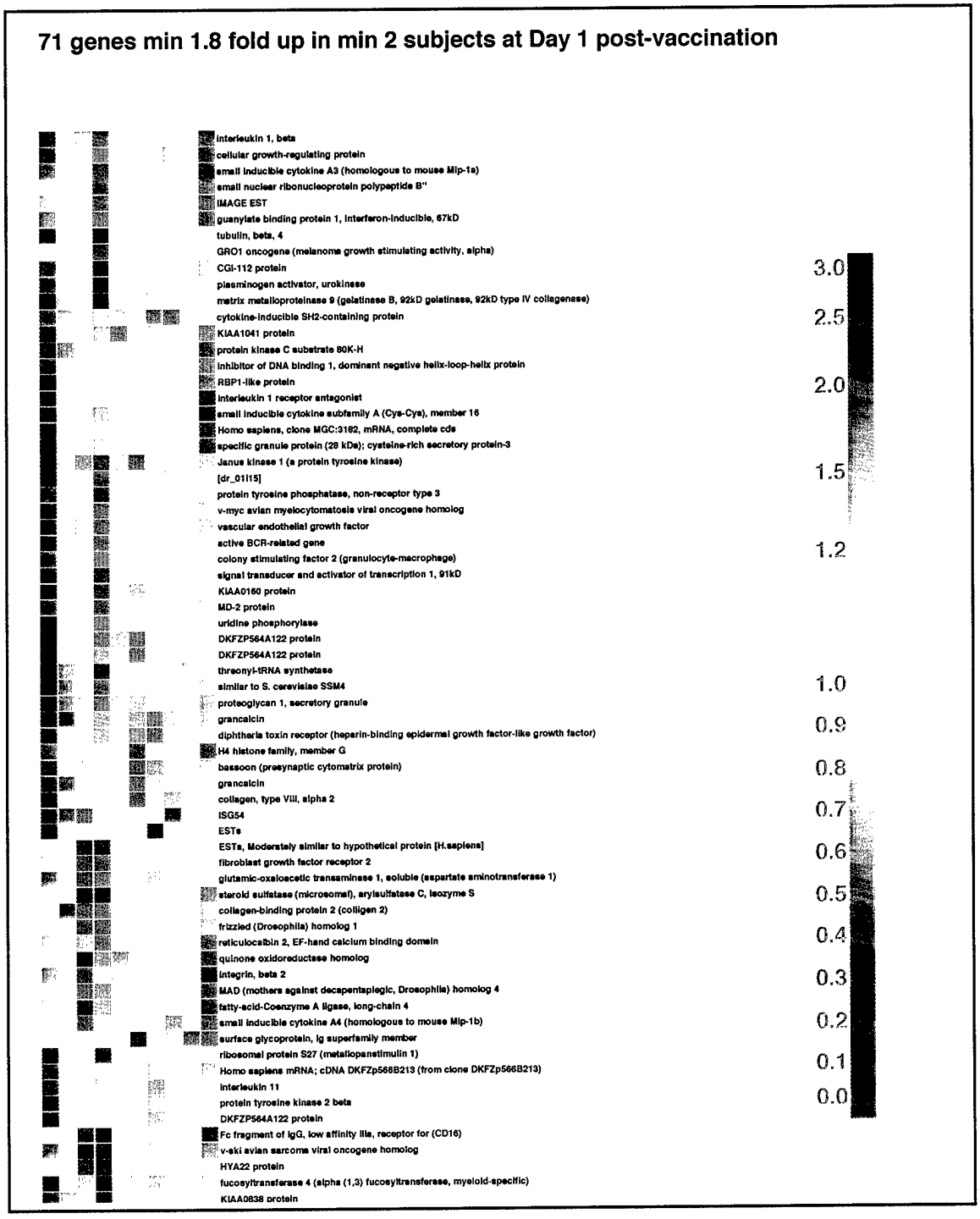

Figure 1

Gene expression profiles in RNA isolated from whole blood from 10 vaccinated individuals 1day post vaccination are shown. Red represents most highly expressed. Microarray data was analyzed and Genetree generated by the program GeneSpring.

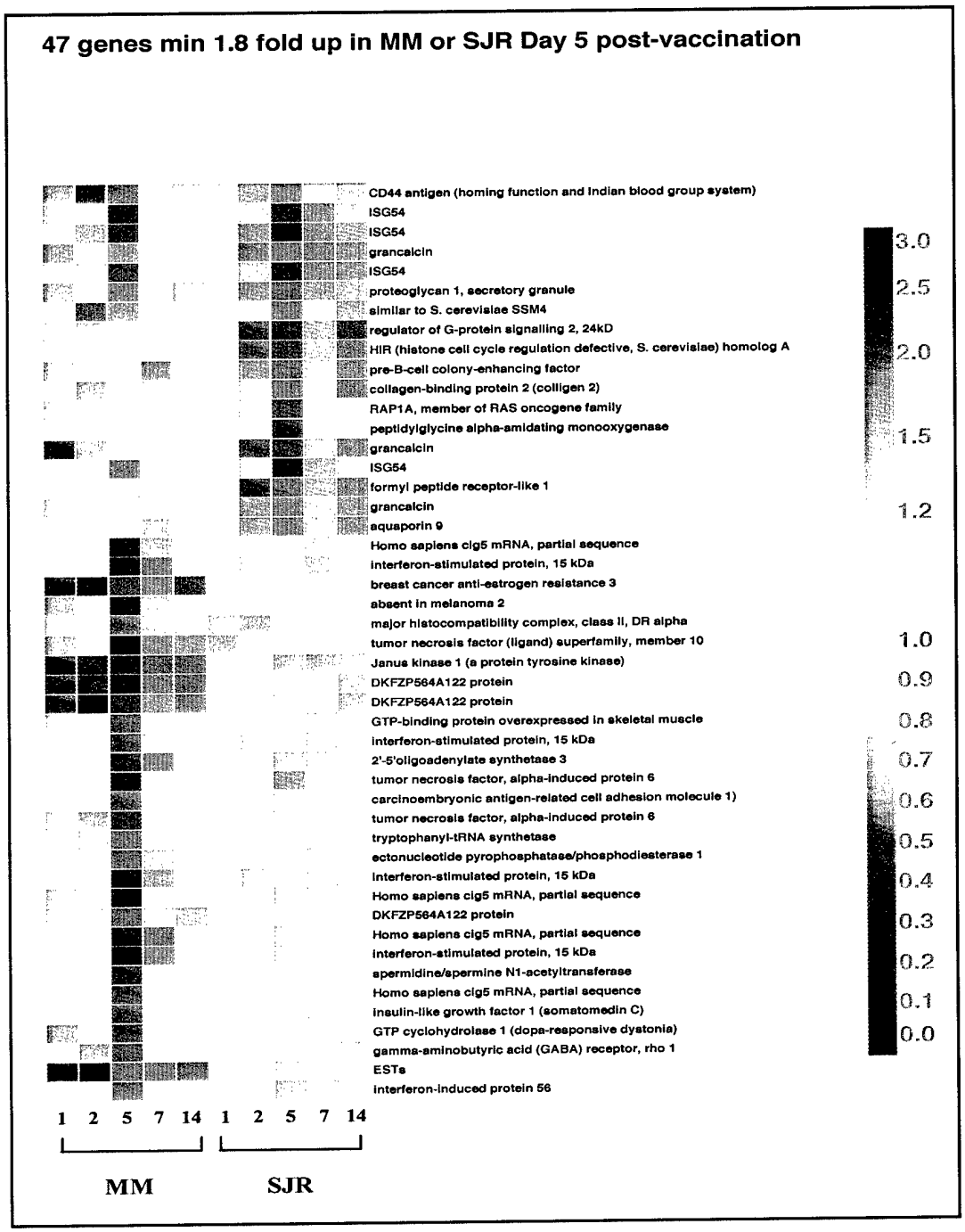

Figure 2. Gene expression profiles at different days in two individuals demonstrating differing clinical reponses to vaccination. Subject MM displayed lymphnode swelling which was absent in subject SJR. A Genetree of genes significantly changed in expression on different days following vaccination (X-axis) is shown.

2. Identifying virus-specific signatures

In general, exposures to biological agents in bioterrorism in humans occur first by the aerosol route with inhalation into the respiratory system. However, the feasibility of using these types of samples for large-scale detection of exposures is unknown. One of our major goals is the identification of early biomarkers of agent exposure in human airway epithelial cells. We are using a lung sample repository that has been maintained in the Lung Biology laboratory over the previous 8 years. Non-trackable sample numbers, which are not linked to the donor, are used as unique non-traceable identifiers. This task has been successful during this interim period in providing the lung epithelial cells in culture, confirming epithelial phenotype, lack of infectious contaminants, and providing epithelial cell RNA in sufficient quantity for microarray experiments using *in vitro* exposure of primary cells to virus (see Zheng S. 2003 appended)

Human airway epithelial cells (HAEC) are grown on collagen-coated dishes, in specialized serum-free media (Clonetics). We have shown that cell cultures of HAEC lead to pure epithelial monolayer cultures. The epithelial nature of cultured HAEC was confirmed by morphology and positive reactions to anti-cytokeratins, which are epithelial cell specific. Non-transformed HAEC are limited to ±25 population doublings, a culture span of about 1-month and a maximum of 4 to 5 passages. All cells were mycoplasma tested and confirmed negative before use in experiments.

We have completed a proof of principle experiment to identify potential sentinel transcripts activated by the presence of viral pathogens infecting HAEC. Cells were grown to approximately 85-90% confluence in 100 mm dishes and infected cells with Influenza A/Japan/305/57, subtype H2N2 (Advanced Biotechnologies, Columbia, MD) which was grown in Madin-Darby canine kidney (MDCK) cells, and suspended in serum-free MEM with Earle's Salts. The virus stock contained $10^{6.25}$ TCID50 (50% of tissue culture infectious dose) per ml. Cells were infected with Influenza A2 Japan virus using 0.2 viral particles/cell for an estimated $8x10^6$ cells/plate. Infection of cells was performed using sterile techniques in the BSL2 virus facility in the tissue culture hood. The virus was added to each plate, transferred to a 37° C incubator and after 1 hour the media and virus was removed from the plates and replaced with 10 ml of fresh media. The cells were returned to the incubator until harvest according to the time course scheduled for each experiment. In addition, control noninfected cells were also harvested at each timepoint as control for microarray analyses. For vaccinia virus infections, the Western Reserve (WR) strain (a gift from R. Condit, Gainesville, FL) was used. Conditions for virus growth, infection, and plaque titration were as described (Condit et al., 1983). BEAS2B and BET1A cells, human bronchial epithelial cell lines transformed by adenovirus12-SV40 virus and SV40 T-antigen respectively (Reddel, et al. 1988) or human airway epithelial cells (HAEC), 399 and 585, cells were inoculated with virus at a multiplicity of infection (MOI) of 10 and incubated at 37C for 30 min in PBS. The inoculum was removed and replaced with fresh medium and the infected cells were incubated at 37C. Cells were harvested by centrifugation and resuspended in PBS. RNA was prepared using phenol/chloroform purification (influenza) or trizol (vaccinia virus) extraction methods and RNA concentrations and quantities were determined for each of the samples, and quality monitored on agarose gels or

by Agilent RNA chip. For influenza virus experiments four experiments were completed using cells extracted from 4 different human donor lungs. Total RNA extracted from plates was 11± 4 ug/1x10^6 cells. RNA was of good quality with no degradation as evaluated by gel electorphoresis. Microarray analysis was performed as described and microarray data analyzed using Genespring software. A list of genes significantly changed in expression following influenza infection at any time point in each of the four samples is shown in Table 1.

Table 1

Systematic	Product
ISG_03G10	LIM domain only 2 (rhombotin-like 1)
ISG_02N10	nuclear antigen Sp100
dR_01B10	interferon-induced protein 56
ISG_03P19	2'-5'oligoadenylate synthetase 3
ISG_02M16	nuclear antigen Sp100
ISG_01H08	cytochrome c-1
ISG_01C24	interferon induced transmembrane protein 1 (9-27)
ISG_02G19	2'-5'-oligoadenylate synthetase 2 (69-71 kD)
ISG_02B20	interferon induced transmembrane protein 2 (1-8D)
ISG_01C07	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)-like 1
AU_02p24	cofilin 2 (muscle)
AU_04g11	cofilin 1 (non-muscle)
AU_04l07	luteinizing hormone/choriogonadotropin receptor
dR_01D17	KIAA0246 protein
ISG_02G18	signal recognition particle 9kD
ISG_02K21	eukaryotic translation initiation factor 4E
AU_02d21	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
AU_02h09	DNA polymerase delta, subunit 3
dR_01H19	connective tissue growth factor
AU_02f21	Interleukin 8
ISG_03A24	diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)
ISG_02H22	interleukin 8

The expression patterns for these genes differs relative to the time after infection (Figure 1). The first 10 genes listed on table 1 are expressed most highly relatively late in infection, the next seven on the list exhibit maximum expression 8 hours after infection while the remaining four are early indicators of influenza virus infection of airway epithelial cells.

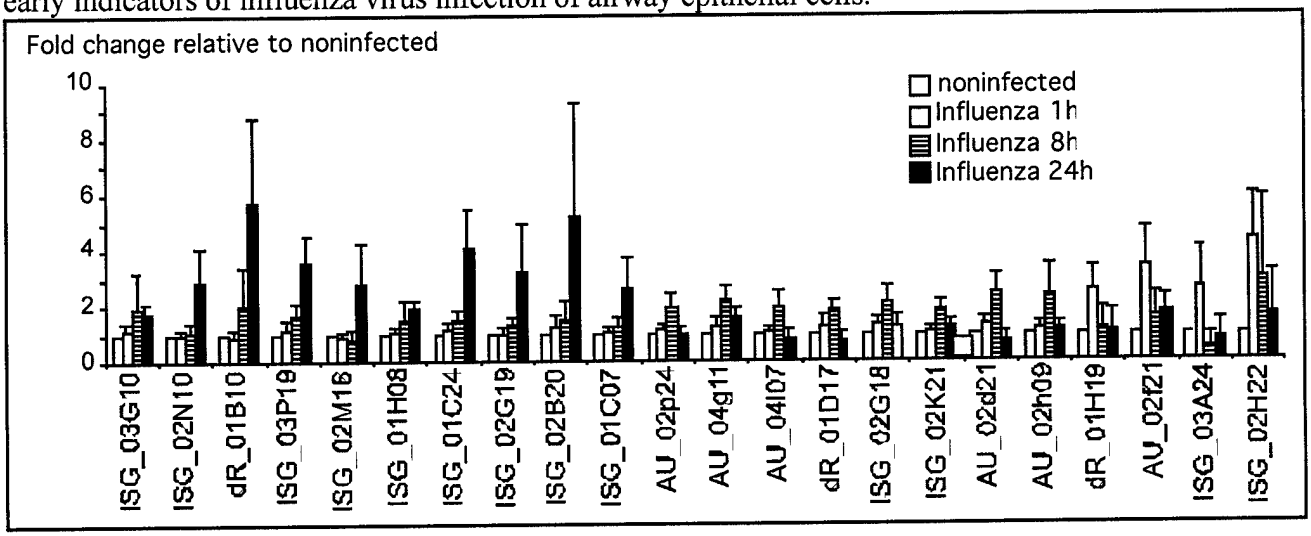

Figure 3. Relative expression levels of selected genes expressed in HAEC infected with influenza virus

In the case of vaccinia virus, a different group of genes were identified (Figure 2) suggesting that it may be possible to discriminate using gene arrays among common respiratory tract infections such as influenza and parainfluenza and orthopox infection where the early clinical symptoms are similar.

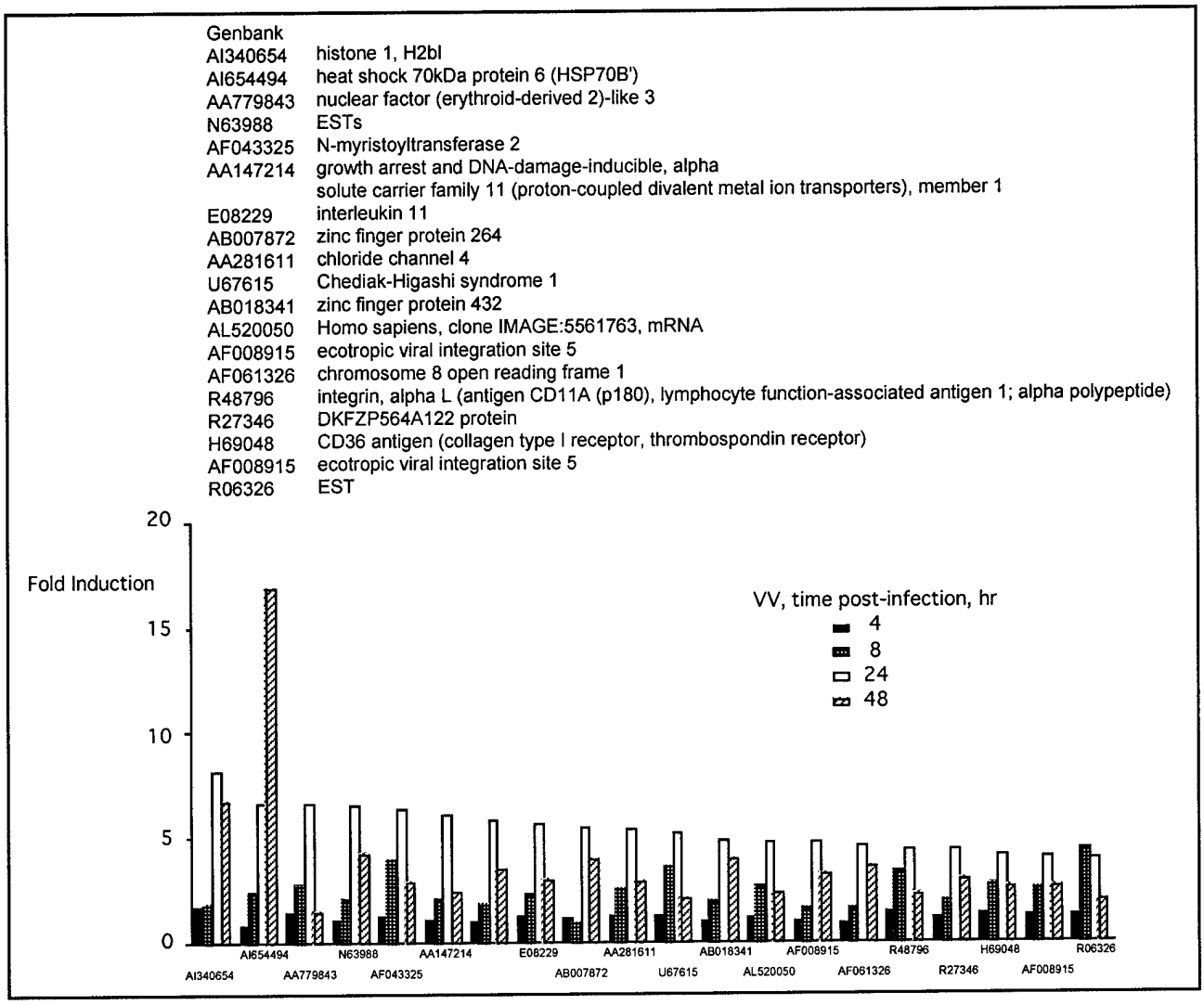

Figure 4. Relative expression levels of selected genes expressed in HAEC following vaccinia virus infection.

3. Descriminating among different viruses infecting lung epithelial cells using transcript profiling

The finding that influenza and vaccinia virus infected airway epithelial cells gave very different gene expression profiles encouraged us to use the IAD array to identify a vaccinia virus specifc gene expression signature using data from vaccinia virus, influenza virus and parainfluenza virus infected HAEC. Briefly a list of 25 genes (Table 2) was derived in Genespring 6.0 using the class prediction function in which you can ask for the minimum number of genes which permit

100% success in assigning viral identity to a set of gene expression profiles from viral infection experiments. A set of profiles representing 19 different hybridisations including both biological and technical replicates of vaccinia influenza and aprainfluenza virus infected HAEC were used. The data for those 25 genes are sufficient to correctly assign viral identity to each profile.

Table 2

Systematic	Genbank	Description
dr_01b18	Hs.1908	proteoglycan 1, secretory granule
dr_01i02	Hs.83758	CDC28 protein kinase 2
dr_01j07	Hs.47572	ESTs, Weakly similar to ORF YKR079c [S.cerevisiae]
au_01m10	no ID	v-jun avian sarcoma virus 17 oncogene homolog
dr_01i07	Hs.75862	MAD (mothers against decapentaplegic, Drosophila) homolog 4
isg_03n01	AA436568	ribosomal protein S4, X-linked
au_02m01	no ID	v-jun avian sarcoma virus 17 oncogene homolog
au_02m13	AB001106	glia maturation factor, beta
isg_03h05	AI351740	lymphotoxin beta (TNF superfamily, member 3)
isg_02k04	AI129113	XIAP associated factor-1
au_03j01	no ID	Glyceraldehyde-3-phosphate dehydrogenase
au_02g12	AF012072	eukaryotic translation initiation factor 4 gamma, 3
au_01h05	X54993	TATA box binding protein
isg_01m15	AA425664	catechol-O-methyltransferase
isg_03g17	AA961735	macrophage myristoylated alanine-rich C kinase substrate
isg_01m04	AA464600	v-myc avian myelocytomatosis viral oncogene homolog
isg_01o24	AI682350	small nuclear ribonucleoprotein polypeptide A
au_02c13	AF001846	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
au_03a02	J03804	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform
dr_01j06	Hs.4084	KIAA1025 protein
au_02f21	Y00787	Interleukin 8
au_02k06	U77352	MAP-kinase activating death domain
au_02n16	U70136	megakaryocyte stimulating factor
isg_02o07	AA677522	small inducible cytokine A3 (homologous to mouse Mip-1a)
isg_02n19	AA608515	Homo sapiens NADH dehydrogenase (ubiquinon)

4. Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis.

The application of the IAD array has allowed us to identify host factors in patients with cystic fibrosis that promote increased virus replication and cytokine production thus providing a mechanism for understanding the severity of virus disease in CF. Increased virus is due to lack of nitric oxide synthase 2 (NOS2) and 2', 5' oligoadenylate synthetase (OAS) 1 induction in response to virus or IFNgamma. This could be attributed to impairment of activation of signal transducer and activator of transcription (STAT)1, a fundamental component to antiviral defense. NO donor or NOS2 overexpression provides protection from virus infection in CF, suggesting that NO is sufficient for antiviral host defense in the human airway and is a potential

strategy for antiviral therapy in CF children. The fact that heterozygous alleles are common in the caucausian population gives cause for concern that these individuals may be at higher risk for viral agents of bioterriorism than the normal population. NO donors may be useful as antivirals in this instance.

B. Assembly and testing of prototype sentinel protein microarrays for detecting signatures of biological agents.

As previously pointed out in our previous progress report to move forward from a theoretical concept to a practical application we needed to assemble the biological agents (or models) and cell cultures, expose cells to the agents, extract protein and purchase and set up materials and equipment. During the second year of study, different cell types infected with a select group of virological and bacterial agents that simulated bioterrorism, biological warfare or were identified as emerging agents were assembled and protein extracted from control and infected cultures. Antibodies specific for detecting activated cell signaling components in response to pathogenic bacterial and viral agents were also assembled or newly derived. Signaling protein signatures for a select group of viruses and bacteria or their products were also determined (see below). We worked on the development of single chain antibodies directed against phosphorylated and non-phosphorylated peptides of key signaling molecules that we have previously identified. As stated in the previous progress report we had difficulties getting positive binders to phospho-peptides which we believed were due to the limited complexity of our home-made scFv libraries. We acquired a large human scFv library (over 10^{11} members) from Cambridge Antibody Technologies (CaT) (Cambridgshire, UK) in an attempt to remedy this problem. CaT is one of the leading phage display, single chain antibody biotechnology companies in the world and has created libraries of antibody genes collected from the blood of healthy individuals. These libraries contain the genes for over 100 billion distinct antibodies. CaT has used these gene libraries as the basis for the production of phage antibodies, created by phage display. The larger the antibody libraries, the more chance there is of them containing high quality antibodies that will bind to any given target molecule. We used the large BMV CaT scFv library to screen a number of the non-phospho and phospho peptides and have been able to generate positive binding scFvs for non-phospho peptides but have had difficulty in generating any additional useful phopho-specific antibodies so far tested. We have not succeeded in using ribosome display technology to increase the avidity of two of these binders so far tested. To solve this roadblock in our progress, we propose to use chain-shuffling to isolate more avid binders. Chain shuffling simply allows one to go back to a population of binders (even weak binders), re-PCR the variable light and heavy antibody chain regions as individual fragments and then again using PCR stitch them together as a scFv-encoding DNA and reclone them to make a new library. This "selected" new library will then be used to select out more avid binders. This technique is commonly used and almost always produces good to strong binders however, it is much more labor intensive than what we currently have been doing in that one must make a new 'selected" library for every target.

To increase our throughput we have gone to selecting multiple targets (non-phosphorylated) simultaneously and it appears to work well with up to 4 targets and we believe that we can

successfully apply this strategy to the phospho-targets as well and reduce the construction of "selected" libraries by a factor of 3 to 4. To additionally increase our throughput we will use a colony picker- robotic gridder to manipulate and array the "selected" libraries so that individual clones can be identified after one to two rounds of selection (see deWildt et al., 2001). The colony picker/ arrayer is available in the Department as part of a shared equipment grant to Dr. DiDonato from the NIH.

The other focus of this task was to identify the molecular signaling pathways activated by bacterial and viral pathogens and their products in a number of relevant cell types (lung epithelial, intestinal epithelial, oral epithelial cells and macrophages). We have had good success in identifying the major stress-activated pathways in lung epithelial cells and in intestinal epithelial cells (see Bose et al., in the appendix). As a by-product of these studies, we have also found that flagellin is the main proinflammatory mediator in both intestinal epithelial cells and also in lung epithelial cells (Tallant et al.,submitted). We have investigated the response of intestinal epithelial cells to the pathogenic bacteria Salmonella and also described the proinflammatory signaling pathways triggered by exposure to flagellin, a potent pro-inflammatory mediator. This finding is of particularly keen interest since flagellin potentially can be used as a potent biological weapon simply by adding a cell-permeating peptide sequence (~13 amino acids) allowing it to pass into and through epithelial barrier cells which the natural flagellin protein does not normally do. This modified protein could be made in vast quantities at an extremely low cost, is easily purified to almost homogeneity and is heat stable allowing it to be administered either in food or water or aerosolized, it is also quite stable in the environment and therefore could be considered a potent "poor man's" biological weapon and its use as such must be considered as a viable threat so the more detailed information we have about its effect on cells, the more effective our responses to neutralize its activity will be.

In addition to using the bacterial pathogen Salmonella typhimurium and the bacterial protein flagellin (described above) to stimulate responses in tissue culture cells and catalogue their signaling pathways we have also studied signaling in response to the lung trophic viruses Respiratory syncitial virus (RSV), human parainfluenza virus (HPIV-3, see previous section on gene arrays), a negative strand RNA virus vesicular stomatitis virus (VSV), and another RNA virus, influenza A (see previous section on gene arrays). These studies have identified a novel anti-viral innate host response that requires the activation of the NF-κB transcription factor and expression of one of its unknown target genes. Failure to induce the production of this unknown anti-viral protein turns a mildly cytopathic virus (HPIV-3) into a highly virulent one similar to RSV (see PNAS paper in appendix). These findings are significant since it demonstrates how a mild cytopathic virus can be turned into a virulent one without genetic manipulation. These results also point to the existence of a factor(s) that can negate the effects of virulent negative strand RNA viruses and likely will have broad-spectrum protective effects among a number of current Category A biologicals. In preliminary studies, the envelope glycoprotein HN of HPIV-3 has been identified as the viral protein recognized by one of the Toll-like receptors (TLRs, either 7 or 8) expressed by the host cell. TLRs 6, 7 & 8 also play an important role in activating the anti-viral state triggered by HPIV-3 infection. A manuscript addressing these findings is presently in preparation. Additional activated signaling pathways in the lung and intestinal

epithelial cell (including ER stress, JAK-STAT pathway proteins and growth factor) in response to viral and bacterial challenges are presently under study.

Key research accomplishments

- Successfully used arrays constructed and tested during the previous granting period to analyze gene expression patterns in RNA extracted from whole blood from subjects undergoing orthopox vaccination against smallpox.
- Established that infection of HAEC by different viruses results in virus-specific gene expression profiles.
- Established a gene expression profile that can distinguish orthopox infection from commonly occurring respiratory infections.

Reportable outcomes

Zheng S, De BP, Choudhary S, Comhair SA, Goggans T, Slee R, Williams BR, Pilewski J, Haque SJ, Erzurum SC.(2003) Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis.Immunity;18(5):619-30.

Bose S, Kar N, Maitra R, **DiDonato JA**, Banerjee AK. (2003). Temporal activation of NF-kappaB regulates an interferon-independent innate antiviral response against cytoplasmic RNA viruses. Proc Natl Acad Sci U S A. **100**:10890-10895.

Conclusions

A custom array specifically designed to detect changes in cellular gene expression resulting from infection by viruses of bacteria has been tested in a live orthopox virus vaccination study. While individual variations were quite marked, adverse events could be detected as a change in profile. This study established that RNA can be extracted from small (2-5ml) quantities of whole blood and exposure to a smallpox surrogate detected using our IAD array. Furthermore, the utility of the array in detecting early adverse events in small samples of whole blood was demonstrated. While we noted that there are cell line specific responses in gene expression profiles to infection with the same virus, the infection of primary airway epithelial isolated from different individuals infected with the same virus gave very similar results suggesting that the individual variation seen in RNA isolated from whole blood was not a major factor in primary airway epithelial cells. A gene expression profile has been established that yielded 25 genes that successfully discriminated orthopox virus infection from other common respiratory viruses. Accordingly we have succeeded in one of our major objectives, which was to define sentinel markers of infection with specific agents. This signature will now be tested in further model experiments in mice (or humans if further vaccinations take place). The development of antibody reagents is continuing with the goal of constructing a prototype protein array over the next funding period.

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Appendices

1. Zheng S, De BP, Choudhary S, Comhair SA, Goggans T, Slee R, Williams BR, Pilewski J, Haque SJ, Erzurum SC.(2003) Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis.Immunity;18(5):619-30.

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APPENDIX 1

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Impaired Innate Host Defense Causes Susceptibility to Respiratory Virus Infections in Cystic Fibrosis

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Summary

Viral infection is the primary cause of respiratory morbidity in cystic fibrosis (CF) infants. Here, we identify that host factors allow increased virus replication and cytokine production, providing a mechanism for understanding the severity of virus disease in CF. Increased virus is due to lack of nitric oxide synthase 2 (NOS2) and 2′, 5′ oligoadenylate synthetase (OAS) 1 induction in response to virus or IFNγ. This can be attributed to impairment of activation of signal transducer and activator of transcription (STAT)1, a fundamental component to antiviral defense. NO donor or NOS2 overexpression provides protection from virus infection in CF, suggesting that NO is sufficient for antiviral host defense in the human airway and is one strategy for antiviral therapy in CF children.

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disorder among Caucasians, affecting an estimated 30,000 persons in the US (Cystic Fibrosis Foundation, 2000). The gene responsible for CF (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989) produces the cystic fibrosis transmembrane conductance regulator (CFTR), a polypeptide of 1480 amino acids with molecular mass of 168 kDa, and function of a cAMP-dependent Cl^- channel (Anderson et al., 1991; Sheppard and Welsh, 1999). CF is characterized by chronic lung infections with bacteria, mostly *Pseudomonas aeruginosa*, intense neutrophil-dominated airway inflammation, and progressive lung disease, which is the major cause of morbidity and mortality. Bacterial colonization of CF lung is usually established in the first decade of life (Rosenfeld and Ramsey, 1992). Little is known about the factors associated with initial colonization in CF lung, but viral infections predispose CF lung to bacterial colonization. Although chronic bacterial infection occurs in older CF children, 39% of CF children in the first year of life are hospitalized with respiratory compromise related to respiratory virus infection. Furthermore, individuals hospitalized with respiratory symptoms during infancy are six times more likely to acquire *Pseudomonas aeruginosa* during early childhood (Armstrong et al., 1998). Studies show a relationship between viral respiratory tract infection with respiratory syncytial virus, parainfluenza virus, and influenza virus and pulmonary exacerbation and disease progression in CF children (Hiatt et al., 1999; Hordvik et al., 1989; Petersen et al., 1981; Wang et al., 1984). Although CF patients have no higher incidence of viral infection, severity of viral infection is amplified.

The innate antiviral response of human cells involves distinct cellular programs (Iordanov et al., 2001). In the presence of dsRNA, a common viral intermediate, 2′, 5′ oligoadenylate synthetase (2′, 5′ OAS), and dsRNA-dependent protein kinase (PKR) promote inhibition of host cell protein synthesis by activating RNase L to degrade viral and cellular RNA and by phosphorylating the α subunit of translation initiation factor, eIF2, to block its recycling from an inactive form, respectively. This prevents viral replication, eventually leading to the self-elimination of the infected cell via apoptosis. This program is probably most efficient for viral infections that are initiated by a small number of infected cells at a local site of virus entry. A second program is the production of antiviral interferons (IFN) by mucosal cells and serves the purpose of preparing adjacent naive cells for resistence to viral invasion. This program requires survival of infected cells and expression of antiapoptotic genes through activation of nuclear factor-κB (NF-κB) transcription factor. NF-κB and interferon regulatory factors (IRF) 3 and 7 are required for production of type 1 interferons (Grandvaux et al., 2002). Subsequently, IFN induces antiviral pathways including PKR, 2′, 5′ OAS/RNase L system, and Mx proteins (Samuel, 1991; Stark et al., 1998). Mx proteins are IFN-inducible, high-abundance GTPases which interfere with viral replication, impairing the growth of negative-strand RNA viruses at the level of viral transcription and other steps (Stark et al., 1998). dsRNA or IFN-γ are also potent activators of nitric oxide synthase 2 (NOS2)- and nitric oxide (NO)-dependent antiviral pathways. High-level NO synthesis results in a large variety of reactive products, which can inhibit viral replication by modifying a number of target molecules essential for replication (Biron, 1999). STAT1, a member of a family of proteins that transduce signals from cell surface receptors to the nucleus and activate transcription by binding directly to regulatory DNA elements, is essential for host antiviral defense. IFN-α and IFN-γ lead to phosphorylation of STAT1 and binding to unique elements in a number of IFN-stimulated genes (ISGs), activating transcription (Haque and Williams, 1998). Although many antiviral genes are induced or activated in direct response to viral dsRNA, fundamental components of antiviral defense are activation of PKR, 2′, 5′ OAS, and NOS2 via the IFN/STAT1 pathways. In support of this, STAT1-deficient mice, which display

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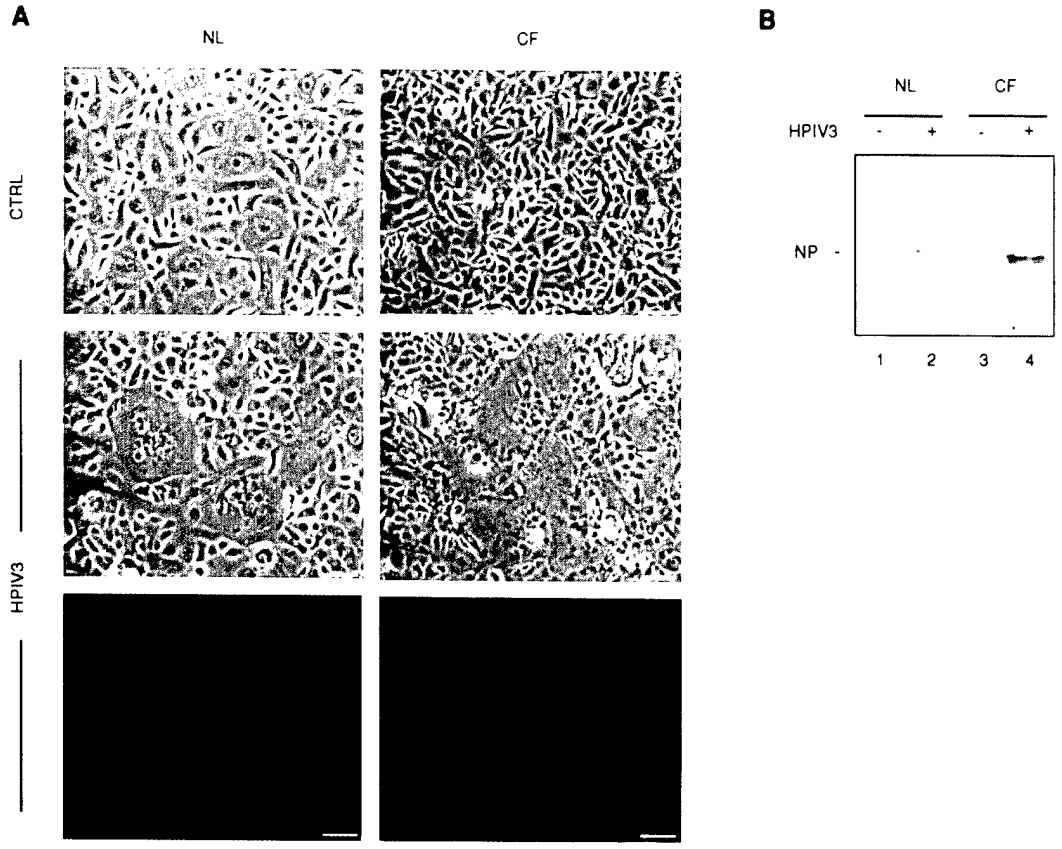

Figure 1. Increased HPIV3 Replication in CF Cells

(A) Phase contrast picture of NL and CF cells, uninfected (upper panels) or 24 hr after HPIV3 infection (middle panels) and immunoflurescence staining for HPIV3 NP 24 hr postinfection (lower panels) (n = 3). Bars, 100 μm.

(B) Equal amounts (20 μg) of ^{35}S-methionine-labeled new protein synthesized in NL and CF cells were immunoprecipitated by HPIV3 anti-RNP antibody and loaded in each lane (n = 3).

a complete lack of responsiveness to IFN, are highly sensitive to infection by virus (Durbin et al., 1996; Meraz et al., 1996).

In this context, we hypothesized that CF airway epithelial cells may be less effective in eliminating viral infection due to an impairment of the antiviral host defense mechanisms in CF lung. Here, we show that CF airway epithelial cells allow increased replication of parainfluenza virus and an increased production of proinflammatory cytokines. Investigation of the innate and interferon (IFN)-mediated antiviral pathways reveals that the antiviral pathway of nitric oxide synthesis is absent in CF. Furthermore, upregulation of 2′, 5′ OAS1 does not occur in CF cells in response to IFN or dsRNA. This can be attributed to impaired STAT1 activation, which may be a central mechanism responsible for the deficiencies in CF antiviral host defense.

Results

Increased Viral Replication in CF

CF and normal (NL) human airway epithelial cells (HAEC) were infected with human parainfluenza virus 3 (HPIV3) (0.1 moi) and syncytia (cell-cell fusion) formation evaluated (Figure 1A). Cell-cell fusion was increased in CF cells compared to NL 24 hr after infection (middle panel). Immunofluoresent staining for HPIV3 N-protein (NP) revealed greater size and number of syncytia containing virus in CF cells (lower panel). To confirm that the NP present in the cell lysate was from viral replication and not from added virus, new protein synthesized was evaluated by ^{35}S-methionine incorporation followed by SDS polyacrylamide gel electrophoresis of cell lysates immunoprecipitated with anti-RNP antibody which recognizes HPIV3 NP. NP was detected at ~2-fold higher level in CF than NL (Figure 1B).

IFN Pretreatment Protects CF Cells from Virus

First identified because of their ability to interfere with virus replication, IFNs are fundamental in host antiviral defense (Biron, 1999; Briscoe et al., 1996; Durbin et al., 1996; Grandvaux et al., 2002; Isaacs et al., 1957; Karaghiosoff et al., 2000; Karupiah et al., 1993; Samuel, 1991; Stark et al., 1998). To investigate IFN antiviral effects in CF, CF cells were pretreated with 1000 U/ml IFN-α, IFN-γ, or no cytokine for 24 hr, and then infected with HPIV3 (0.1 moi). Syncytia formation was prevalent in untreated CF cells (Figure 2A, upper-right panel), but pretreatment with IFN-α or IFN-γ prevented viral syncytia formation (Figure 2A, lower panels). Evaluation of HPIV3 N-mRNA expression revealed that more virus N-mRNA was formed in infected CF than in NL cells,

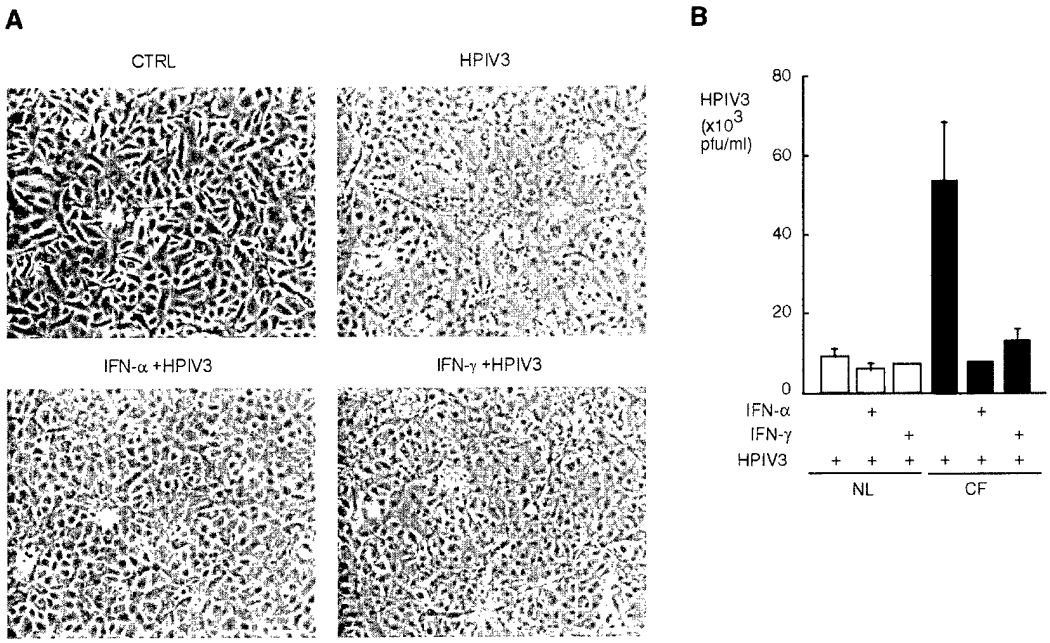

Figure 2. IFN Pretreatment Protects CF Cells from HPIV3 Infection

(A) Phase contrast pictures of CF cells, untreated (upper-left panel), infected with HPIV3 (upper-right panel), or pretreated with IFN-α (lower-left panel) or IFN-γ (lower-right panel) 24 hr before HPIV3 infection (n = 2). Bars, 100 μm.

(B) Infectious viral particles in media overlying cells untreated or pretreated with IFN-α (1000 U/ml) or IFN-γ (1000 U/ml) measured by plaque assay [plaque forming units (pfu)/ml × 10^3] after HPIV3 infection (0.1 moi). Infectious viral particles are higher titer in media overlying CF cells (n = 5) than NL (n = 3) [p = 0.015].

and IFN-α or IFN-γ pretreatment significantly reduced the N-mRNA in both NL and CF cells (data not shown). Media overlying cells were evaluated for infectious HPIV3 particles by plaque assay. CF produced ~6-fold more infectious HPIV3 as compared to NL (CF: 53 ± 15, range 30~70, n = 5; NL: 8 ± 2, range 6~10, n = 3 [×10^3 pfu/ml]). IFN-α and IFN-γ pretreatment reduced virus in CF to NL levels (Figure 2B). Innate antiviral pathways in NL cells appeared effective in eliminating viral replication, but IFN pretreatment reduced viral load by ~1.5-fold. IFN-α and IFN-γ pretreatment reduced virus in CF by 7- and 5-fold, respectively ($p < 0.05$, student's t test).

Increased viral replication may result in an increase in proinflammatory cytokine production and contribute to severity of virus infection in vivo (Matsukura et al., 1996; Zhu et al., 1996). Thus, cytokine production by cells was evaluated. Supernatant from CF cells 24 hr after HPIV3 infection had higher IL-6 and IL-8 compared to NL, although baseline levels were similar [(baseline: IL-6 pg/ml, CF 13 ± 1, NL 12 ± 2; IL-8 pg/ml, CF 195 ± 87, NL 164 ± 30; n = 3, $p > 0.05$ CF versus NL), (24 hr postinfection: IL-6 pg/ml, CF 2568 ± 1996, NL 208 ± 62; IL-8 pg/ml, CF 11920 ± 8606, NL 2822 ± 245, n = 3, $p < 0.05$, 24 hr comparison, CF versus NL, Mann-Whitney test)].

Expression of Antiviral Proteins in CF

CF and NL cells infected with HPIV3 (0.1 moi) or treated with IFN-α for 24 hr expressed MxA. IFN-α induced higher MxA compared to HPIV3, while IFN-γ did not induce MxA (Figure 3A). MxA was produced at later times after HPIV3 infection as compared to IFN-α stimulation (data not shown). IFN-α is synthesized by lung epithelial cells after viral infection (Gao et al., 1999), and virus-induced MxA expression is likely a consequence of IFN-α (Pavlovic et al., 1992; Ronni et al., 1997). Similar levels of IFN-α were produced by CF and NL in response to virus, reaching peak levels in media overlying cells by 6 hr postinfection (data not shown).

Western analyses for IRF-1, PKR, RNase L, and 2′, 5′ OAS1 were performed with cell lysates collected at 4, 16, and 24 hr after stimulation with virus mimic, dsRNA, or IFN-γ. PKR and IRF-1 were induced by IFN-γ and polyIC in both NL and CF. RNase L did not change before or after stimulation but was present in both cell types. Although NL cells increased 2′, 5′ OAS1 after stimulation, CF cells failed to upregulate expression of 2′, 5′ OAS1 (Figure 3B). Viral induction of NOS2 in CF and NL was assessed 24 hr after HPIV3 infection (0, 0.2, 0.4, 1.0 moi). NL showed a dose-dependent induction of NOS2 by HPIV3, but CF had no detectable NOS2. Expression of MxA confirmed the presence of viral infection (Figure 3C). Reverse transcription of RNA and polymerase chain reaction of cDNA (RT-PCR) analysis of NOS2 mRNA in CF and NL confirmed lack of NOS2 induction in CF in response to HPIV3 (data not shown).

Early in virus infection, host defenses including NOS2 may be induced by dsRNA through PKR signaling pathways, independent of IFN-γ, in NL cells (Uetani et al., 2000). However, by 24 hr after infection, large amounts of IFN-γ are produced which lead to activation of numerous downstream target genes. Specifically, IFN-γ is a

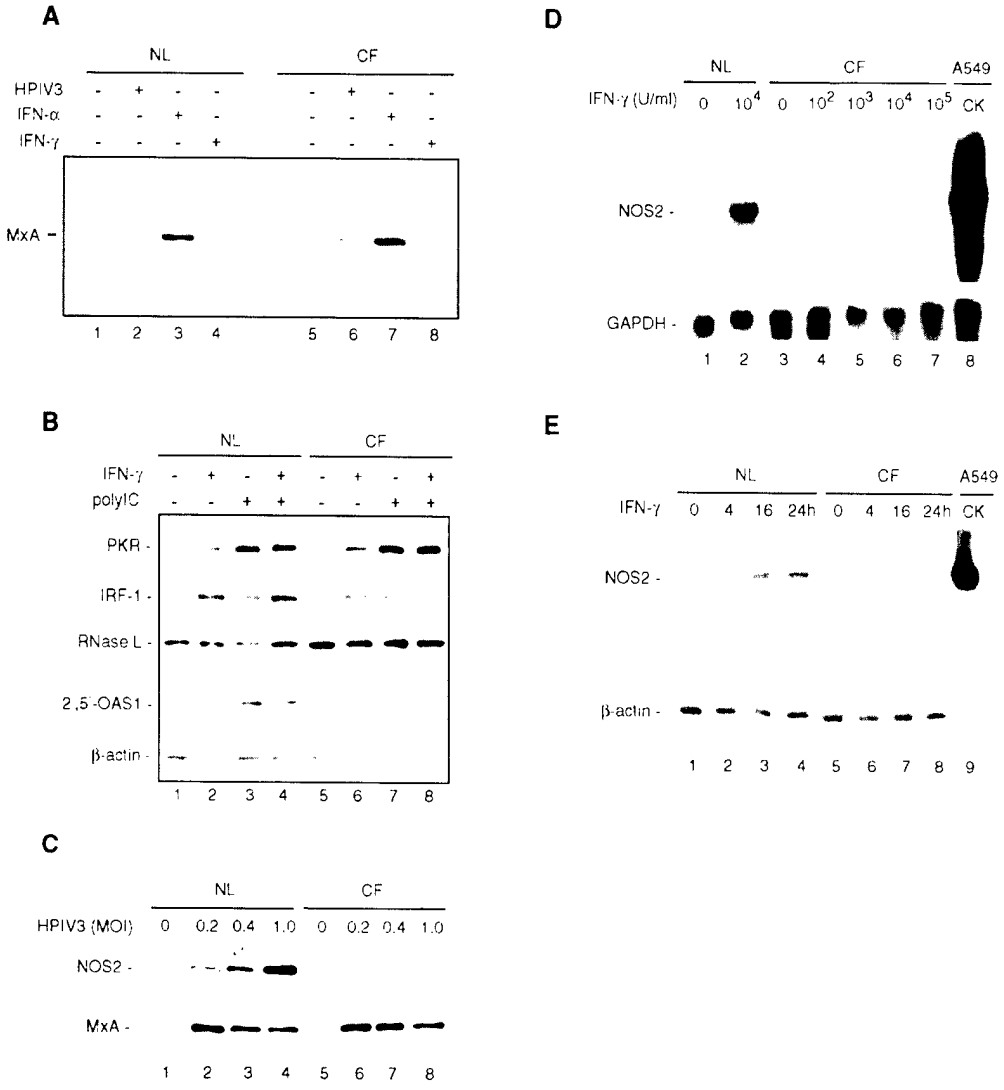

Figure 3. Impaired Antiviral Pathways in CF Cells

(A) Western analysis of MxA in CF and NL cells, untreated, infected with HPIV3 (0.1 moi), or stimulated by IFN-α (1000 U/ml) or IFN-γ (1000 U/ml) for 24 hr (n = 2).

(B) Western analysis of PKR, IRF-1, RNase L, and 2′, 5′- OAS1 in CF and NL cells, untreated, or treated with IFN-γ (1000 U/ml), polyIC (100 ng/ml), or by mixture of IFN-γ and polyIC (n = 3).

(C) Western analysis for NOS2 and MxA in NL and CF cells, uninfected and infected with HPIV3 (n = 2).

(D) Northern analysis for NOS2 in total RNA (4 μg/lane) from CF or NL cells 24 hr after IFN-γ stimulation. Total RNA (5 μg/lane) from A549 cells 8 hr after stimulation with 10^3 U/ml IFN-γ, 0.5 ng/ml IL-1β, and 10 ng/ml TNF-α (cytokine mixture, CK) was used as positive control (n = 2).

(E) Western analysis of NOS2 protein in cell lysate (50 μg total protein/lane) from NL or CF cells 24 hr after IFN-γ stimulation (n = 3).

potent inducer of NOS2 gene expression in normal human airway cells (Guo et al., 1997; Uetani et al., 2000). Here, Northern analysis of NOS2 expression revealed that NL cells expressed NOS2 mRNA upon IFN-γ exposure, while CF cells did not (Figure 3D). Western analysis of proteins extracted at different time points after IFN-γ stimulation showed that NL produced NOS2 protein as early as 16 hr, while CF had no detectable NOS2 (Figure 3E). We also tested induction of NOS2 by polyIC, and combinations of cytokines (IFN-γ, IL-1β, TNF-α) in replicate experiments (n = 3). NOS2 was not induced in CF cells by any combination of stimuli (data not shown).

Similar IFN Response in CF and NL Cells

Based upon findings of defective induction of two antiviral pathways, we expanded evaluation of the IFN response in CF. We compared gene expression profiles in CF and NL at baseline (Figure 4A) and 8 hr after IFN (Figures 4B and 4C) by a custom-constructed ISG/AU/dsRNA cDNA microarray, which contains 2921 genes specific for viral and IFN responses. IFN responses were similar between CF and NL with only 0.9% and 0.5% difference in IFN-α- and IFN-γ-induced changes in gene expression (correlation of CF to NL response: IFN-α R^2 = 0.931; IFN-γ R^2 = 0.940). IFN-α induced 81 genes and

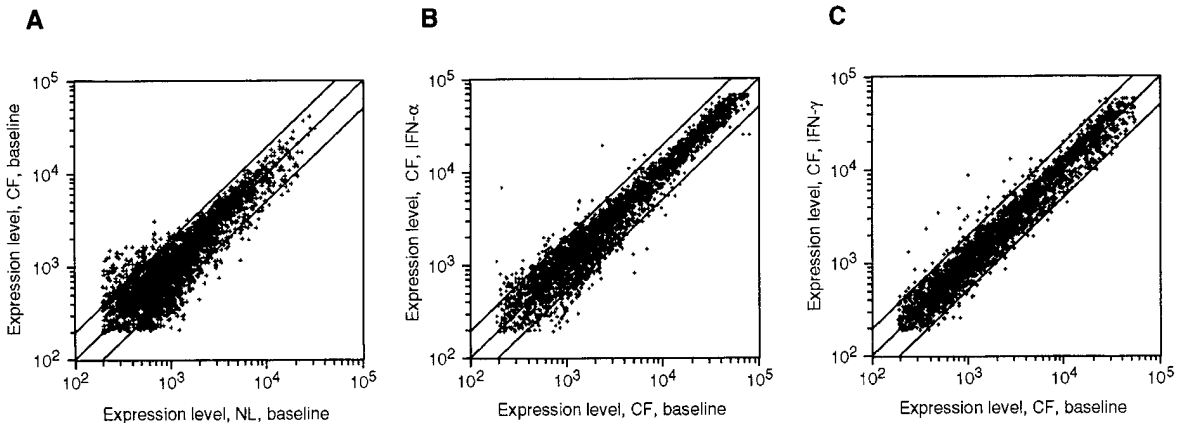

Figure 4. Gene Expression Profile of CF and NL Cells

(A) Baseline gene expression of CF cells compared to NL. (B) Gene expression 8 hr after IFN-α or (C) IFN-γ treatment in CF cells.

repressed 68 genes; IFN-γ induced 27 genes and repressed 33 genes. This similarity of CF response to NL accounts for the effectiveness of IFN pretreatment in inhibiting virus replication in CF cells. On the other hand, a baseline comparison between CF and NL evaluated by ISG/AU/dsRNA microarray identified 226 differentially expressed genes. In CF cells, 136 genes (4.6% of total genes) were 2-fold upregulated, and 90 genes (3% of total genes) were 2-fold downregulated as compared to NL. This baseline difference was confirmed by transcriptosome analysis on Affymetrix HG-U133A GeneChips. Table 1 highlights the genes that are different (≥1.2-fold change) and relevant to IFN, antiviral effects, and/or NOS2 induction. Notable findings include decreased JAK1, a receptor-associated kinase essential for IFN signaling, and increased IRF2, a competitive inhibitor of IRF-1. Both genes are key to antiviral defense and specifically to NOS2 induction (Briscoe et al., 1996; Kamijo et al., 1994; Nelson et al., 1993). The 2′, 5′ OAS1 was also lower in CF at baseline, confirming the Western blot analysis (Figure 3B).

Transcription Factors in CF

Further experiments were performed to investigate the mechanism of deficiency of antiviral host defense in CF, and specifically the reduced NOS2 expression in CF. To evaluate signal transduction proteins IRF-1 and NF-κB, which are important to the host antiviral response including NOS2 induction, we treated CF and NL with IFN-γ (10^3 U/ml), tumor necrosis factor-α (TNF-α) (10 ng/ml), or synthetic dsRNA (polyIC) (100 ng/ml) as a mimic of virus infection, then the transcription factor activation

Table 1. Gene Expression in CF Cells Relative to NL at Baseline

UniGene	Gene Description	Ratio CF/NL	Genebank
Cytokine-Related Genes			
Hs.93913	IL-6: interleukin 6	3.1	NM_000600
Hs.624	IL-8: interleukin 8	2.22	NM_000584
Hs.1722	IL-1 α: interleukin 1 α	3.13	M15329
Hs.285115	IL-13 receptor, α 1	1.42	U81380
Hs.25954	IL-13 receptor, α 2	2.26	NM_000640
Hs.196384	PTHS2: prostaglandin-endoperoxide synthase 2	2.59	NM_000963
Hs.372783	SOD 2: superoxide dismutase 2, mitochondrial	2.79	X15132
Hs.211600	TNFAIP3: tumor necrosis factor α 3	1.51	AI738896
Interferon/Virus-Related Genes			
Hs 83795	IRF 2: interferon regulatory factor 2	1.33	NM_002199
Hs 115541	JAK2: Janus kinase 2	2.00	AF001362
Hs.86958	IR-2: interferon receptor 2	>2	L41944
Hs.179972	IFI α: interferon α-induced protein	1.30	NM_018011
Hs.50651	JAK 1: Janus kinase 1	<0.5[a]	
Hs.82396	2′, 5′ OAS1: 2′, 5′-oligoadenylate synthetase 1	0.43	NM_002534
Apoptosis			
Hs.381231	caspase 8	>2	NM_00128
Hs.9216	caspase 7	1.75	NM_001227
Others			
Hs.234642	APQ3: aquaporin 3	0.47	NM_004925
Hs.89603	MUC1: mucin 1	0.37	NM_002456

[a] Ratio from cDNA microarray data. Gene expression level is below detection limit on Affymetrix genechip. All other ratios are from Affymetrix genechip.

analyzed in whole-cell extract (WCE) by electrophoretic mobility shift assay (EMSA). In contrast to previous reports of reduced IRF-1 expression in whole lungs of CF mice (Kelley and Elmer, 2000; Widdicombe, 2000), IRF-1 was strongly activated by IFN-γ in both CF and NL. Its activation by TNF-α or polyIC was weaker but similar in CF and NL (Figure 5A). Similarly, NF-κB was activated by dsRNA or TNF-α in both CF and NL (Figure 5B). Quantitation of total NF-κB (p65 and p50) showed no difference between CF and NL (NF-κB relative units: nonstimulated, CF 990 $\pm$ 380, NL 1090 $\pm$ 360; IFN-γ, CF 970 $\pm$ 490, NL 1240 $\pm$ 350; polyIC, CF 2000 $\pm$ 310, NL 2670 $\pm$ 280; TNF-α CF 3250 $\pm$ 140, NL 4130 $\pm$ 790; $n = 3$, all $p > 0.05$).

Activation of STAT1 is essential for NOS2 expression and the antiviral response (Gao et al., 1997; Guo et al., 1997; Heitmeier et al., 1999). To evaluate STAT1, CF and NL were exposed to IFN-γ (10^3 U/ml) for 30 min, then WCE collected and analyzed by EMSA with ^{32}P-labeled GAS oligo duplex. CF had lower STAT1 activation compared to NL (Figure 5C). Impairment of STAT1 activation was consistent in CF, and ~60% of NL (Figure 5D). STAT1 is important for not only NOS2 expression, but also for STAT1 itself. To evaluate STAT1 production in CF, CF and NL cells exposed to IFN-γ for 24 hr were evaluated by Western blot using rabbit polyclonal anti-STAT1 Ab. 2fTGH and U3A, human fibroblast cell lines with and without expression of STAT1 (Muller et al., 1993), were used as positive and negative controls for STAT1 expression. Baseline STAT1 protein in CF was less than NL, and 24 hr after IFN-γ, NL expressed more STAT1 than CF (Figure 5E). STAT1 protein in CF was only 53% of that in NL (CF = 1.6 $\pm$ 0.7, NL = 3.0 $\pm$ 1.3, $n = 4$, $p < 0.05$). Furthermore, after IFN-γ stimulation, STAT1 in CF was significantly lower than NL (CF = 5.1 $\pm$ 0.6, NL = 10.6 $\pm$ 2.0, $p < 0.01$) (Figure 5F).

Overexpression of NOS2 or NO Donor Protects CF from Virus

Previous work suggests that loss of NOS2 expression in cells leads to increased susceptibility to viral infection (Flodstrom et al., 2001; Karupiah et al., 1998; Noda et al., 2001). Induction of NOS2 prior to infection is associated with inhibition of viral replication (Reiss and Komatsu, 1998; Sanders, 1999). Since CF cells are unable to express NOS2, NOS2 expression construct or NO donors were used to correct the NO deficiency. We introduced NOS2-transgene into CF cells by transfecting the cells with NOS2 expression plasmid (pCCF37). Control CF cells were transfected with reverse sequence NOS2 (R-NOS2) plasmid (pCCF38), or liposome reagent without plasmid, or left untreated. All cells were infected with HPIV3 (0.5 moi) 24 hr after transfection. Alternatively, two types of NO donors, S-nitroso-N-acetyl penicillamine (SNAP) or deta NONOate, were added to cells at the time of viral infection. NOS2 was expressed in CF transfected with pCCF37 but not in control CF cells (Figure 6A). Indicative of viral production, HPIV3 NP was present in untreated and control transfected cells but not in CF cells expressing the NOS2 transgene. Quantitated as nitrite and nitrate in the media, NO production in CF cells transfected with NOS2 transgene was similar to levels produced by NL cells stimulated with IFN-γ [$NO_2^- + NO_3^-$ (μM): CF cells + NOS2 transgene, 8.0 $\pm$ 1.0; NL cells + IFN-γ, 9.5 $\pm$ 0.5]. NO donor compounds produced higher levels of NO in the media [$NO_2^- + NO_3^-$ (μM): SNAP, 50 $\pm$ 20; detaNO, 29 $\pm$ 1]. NO donors SNAP and deta NONOate, decreased viral load ~2.5-fold. Strikingly, CF cells transfected with NOS2 transgene (pCCF37) had nearly undetectable infectious virus in the overlying media (Figure 6B). NOS2 overexpression may be more efficient than NO donors because NOS2 transgene provides continuous generation of intracellular NO.

Discussion

Here, CF airway epithelial cells are shown at the cellular level to be more susceptible to HPIV3 infection than NL. Increased virus is due to lack of specific antiviral host defense in CF, including NOS2 and 2′, 5′ OAS 1 which may be attributed to impairment of activation of STAT1. In support of the biological relevance of ~6-fold increase of virus, murine studies have shown that loss of innate host defenses leads to a moderate increase in virus, but significantly more severe clinical outcomes (Flodstrom et al., 2001; Kosugi et al., 2002; Noda et al., 2001; Xiang et al., 2002; Zhou et al., 1999). For example, even though the increase of virus is modest in organs of NOS2-deficient (NOS2 $^{-/-}$) mice with cytomegalovirus (Noda et al., 2001) or coxsackievirus B4 (Flodstrom et al., 2001) as compared to wild-type, the NOS2 knockout mice have higher mortality and decreased virus clearance. Likewise, CMV replication is only moderately enhanced as evidenced by 5-fold increase in viral titers in mice pretreated with a specific inhibitor of NOS2, but this results in viral persistence and latency (Kosugi et al., 2002). Mice triple deficient in Mx, RNase L, and PKR have increased susceptibility to virus, although viral titers are not significantly elevated in tissues (Xiang et al., 2002; Zhou et al., 1999). More severe clinical outcomes with modest increase of virus may occur due to inherent viral properties and/or altered host cellular response (Garcia-Sastre, 2001, 1998; Seo et al., 2002). For example, virulence may be increased with moderately higher titers due to more efficient inhibition of host antiviral pathways. Conversely, greater activation of signaling pathways, such as NF-κB, due to increased dsRNA produced during increased viral replication, may amplify proinflammatory cytokine production (Matsukura et al., 1996; Zhu et al., 1996). In this study, CF cells released more IL-6 and IL-8 than NL in response to virus. Higher levels of IL-6 and IL-8, which are involved in neutrophil accumulation and degranulation and contribute to greater airway inflammation and more severe respiratory symptoms with virus (Matsukura et al., 1996; Zhu et al., 1996). For example, severity of clinical symptoms with rhinovirus is primarily related to high levels of IL-6 in nasal secretions (Zhu et al., 1996). CF airways, even in infants, contain higher levels of proinflammatory cytokines, particularly IL-6 and IL-8, irrespective of bacterial colonization (Aldallal et al., 2002; Noah et al., 1997). Thus, it has been hypothesized that inflammation is intrinsic to the CF neonatal airway prior to infection. Here, baseline IL-6 and IL-8 secretion are similar in CF and

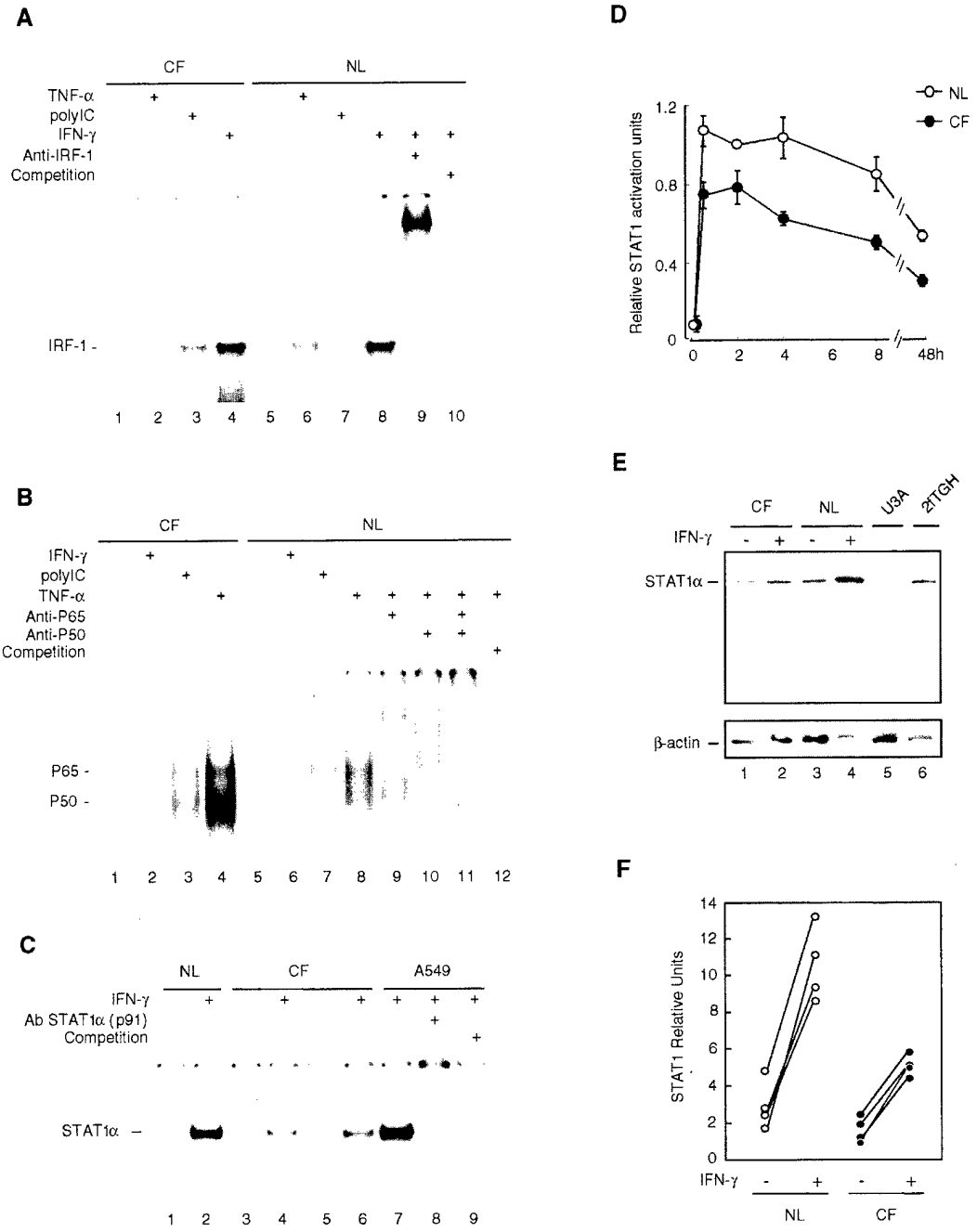

Figure 5. Activation and Expression of Transcription Factors in CF Cells

(A) WCE (4 μg) from CF and NL cells, untreated or treated with TNF-α, polyIC, or by IFN-γ for 3 hr were evaluated for IRF-1 by EMSA (n = 4).

(B) NF-κB activation was evaluated by EMSA in cells stimulated with TNF-α, polyIC, or by IFN-γ for 1 hr (n = 3).

(C) CF and NL cells were stimulated with IFN-γ for 30 min and WCE collected to evaluate for STAT1 activation by EMSA. IFN-γ-stimulated A549 was a positive control, and supershift with anti-STAT1 (p91) and competition with unlabeled GAS probe confirmed presence of STAT1 in the complex.

(D) STAT1 activation at different times was quantitated in four independent EMSA experiments, which were averaged and expressed as relative units normalized to NL value at 2 hr.

(E) Cell lysate (20 μg total protein/lane) from CF or NL 24 hr after IFN-γ stimulation was evaluated for STAT1 (p91) expression by Western analysis. Lysates from 2fTGH and U3A were used as positive and negative controls.

(F) Quantitation of Western analysis of STAT1 expression in cell lysate from four pairs of NL and CF cells, unstimulated or 24 hr after IFN-γ.

NL, but IL-6 and IL-8 mRNA are higher, which may account for the greater release of cytokines upon viral infection. Thus, virus may be one stimulus for the increased cytokine production in CF airways. Taken together, the susceptibility of CF infants to virus may be explained by increased virus and cytokine production,

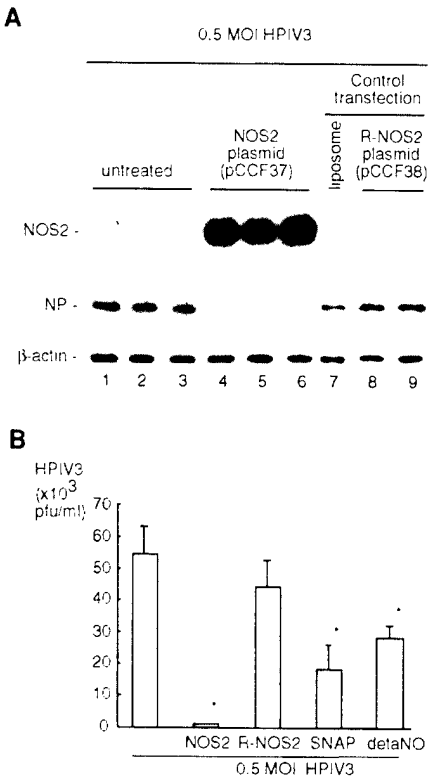

Figure 6. NOS2 Overexpression or NO Donors Protect CF Cells from HPIV3 Infection

(A) Western analysis for NOS2 and HPIV3 NP in CF cells infected by HPIV3, transfected with NOS2 expression construct (NOS2, pCCF37), exposed to reagent alone, or transfected with reverse sequence NOS2 expression construct (R-NOS2, pCCF38) 24 hr prior to infection ($n = 2$).

(B) Plaque assay using media overlying CF cells 24 hr after HPIV3 infection (0.5 moi). 24 hr prior to infection, CF cells were transfected with NOS2 expression construct (NOS2, pCCF37), reverse sequence NOS2 expression construct (R-NOS2, pCCF38), reagent alone (liposome), or left untreated. At the time of infection, some untreated CF cells were exposed to NO donors, SNAP, or deta NONOate (detaNO). Untreated cells have higher titer of infectious virus production than cells with NOS2-transgene or with NO donors [$n = 3$, $^*p < 0.02$].

which results in greater airway inflammation and the severe respiratory symptoms of CF infants with virus infection.

Despite defects in antiviral defenses, pretreatment with IFNs protected CF from virus. The biologic consequences, including antiviral effects, of IFN are mediated by multiple independent genes. Induction of over 600 genes has been identified in response to IFNs (de Veer et al., 2001). Thus, it is difficult to assign IFN antiviral action to any specific gene. Redundancy of antiviral defense is supported by the fact that pretreatment with exogenous IFN leads to a protective antiviral state despite defects in various antiviral pathways. However, it is clear that if the early antiviral defenses are lacking, using a strategy of knockout of specific antiviral genes, virus infection can lead to devastating effects despite the presence of intact IFN pathways (Kosugi et al., 2002; Noda et al., 2001; Xiang et al., 2002; Zhou et al., 1999). For example, mice deficient in Mx, RNase L, and PKR, which are markedly susceptible to viral infections, are nevertheless rescued by pretreatment with IFN (Zhou et al., 1999). Thus, virus-inducible, cell-autonomous innate defenses are important to inhibiting virus, and indeed may be crucial to host defense against viruses with strategies that interfere with IFN signaling, such as HPIV3.

STAT1 is required for IFN signal transduction in the cell and essential for the survival response to virus infection (Durbin et al., 1996; Meraz et al., 1996). Despite numerous downstream targets of STAT1 activation, loss of NOS2 has been identified as a primary factor in the susceptibility of STAT1 null animals to virus (Karupiah et al., 1993). Although not clearly understood, decreased STAT1 also produces a deficient antiviral state and loss of NOS2, while other IFN-mediated genes respond normally (Briscoe et al., 1996; Karaghiosoff et al., 2000). In two prior studies, nonfunctional JAK1 or Tyk2, receptor-associated kinases in the IFN signaling pathway, resulted in decreased STAT1 protein and activation, and a defective antiviral state, although the response to IFN-α or -γ was intact (Briscoe et al., 1996; Karaghiosoff et al., 2000). The Tyk2-deficient cells displayed a phenotype remarkably similar to the CF cells: increased virus replication in cells, impairment of STAT1 activation, with almost all IFN-dependent pathways intact except for NOS2. Altogether, these and the present study suggest that a threshold of STAT 1 may be required for the antiviral state, expression of NOS2, and perhaps other antiviral genes, such as 2′, 5′ OAS. Alternatively, a JAK1- or Tyk2-dependent signal may be required, in addition to STAT1, for expression of NOS2, and for the antiviral state (Briscoe et al., 1996).

Because IFN/STAT1 pathways are so effective in preventing viral infection, many viruses have developed mechanisms to evade the interferon system of the host. All members of the paramyxovirus family interfere with IFN signaling, although by different mechanisms (Andrejeva et al., 2002; Young et al., 2000). HPIV3 inhibits IFN signaling, through specific reduction of serine phosphorylation of STAT1α (Young et al., 2000). Serine phosphorylation is intact in CF (data not shown), but CF cells with impaired IFN activation of STAT1 may be particularly vulnerable to serine phosphorylation block by HPIV3, resulting in more effective interference with IFN signaling. While interference with IFN signaling is a common strategy by which paramyxovirus circumvents antiviral defenses (Andrejeva et al., 2002; Young et al., 2000), viral proteins which block NOS pathways have not been reported. Our data support that HPIV3 may not have specific strategies to escape NO effects. NO inhibits virus replication and even latency of virus, including coxsackievirus, influenza A & B, murine cytomegalovirus, vaccinia, ectromelia, and herpes simplex-1 (Croen, 1993; Flodstrom et al., 2001; Karupiah et al., 1998; Karupiah and Harris, 1995; Rimmelzwaan et al., 1999; Saura et al., 1999; Zaragoza et al., 1997). Here, HPIV3 is also shown to be inhibited by NO. Two specific virus targets of NO, ribonucleotide reductase and viral protease, have been suggested on the basis of in vitro exposure of viral protein to NO donors in cell free systems (Croen, 1993; Lepoivre et al., 1991; Saura et al., 1999). These two known targets are absent in HPIV3. Although viral proteins may be targets of NO, NO also affects host proteins, which is relevant to HPIV3 since it requires host

proteins for transcription and replication (De et al., 1993). Known targets for NO modification include thiol groups and tyrosine, and NO may bind to heme iron in proteins (Grisham et al., 1999). In lung epithelial cells, over 40 cellular proteins are modified by tyrosine nitration, with consequences on activity and function (Aulak et al., 2001). Tyrosine nitration is decreased by NOS inhibitors and in NOS2 knockout cells; thus, NO modification of both host and viral proteins and subsequent effects on protein expression and activity are also likely reduced in CF cells which lack NOS2.

It is interesting to speculate about whether CFTR has a direct effect or is a modifier gene for expression of STAT1, 2′, 5′ OAS1, or NOS2. Inhibition of CFTR function results in reduced NOS2 mRNA in human tracheal epithelial cell lines, while overexpression of human CFTR in CF mice intestinal epithelium leads to NOS2 expression in the ileum (Steagall et al., 2000). These results suggest that NOS2 expression may be directly related to the presence of functional CFTR. In addition, the present findings suggest that STAT1 and NOS2 may be potential gene modifiers of the disease severity in CF lung disease. An important component of the innate host defense in the airway is the ability of respiratory epithelial cells to produce NO continuously in vivo (Sanders et al., 1998). The continuous production of NO in the airways is due in part to expression of NOS2 (Guo et al., 1995). CF infants at birth prior to the onset of respiratory symptoms/infection have exhaled NO 3-fold lower than in healthy controls, suggesting that the defect in NOS2 expression occurs prior to onset of recurrent infections (Elphick et al., 2001). Here, NOS2 is conclusively shown to be sufficient for antiviral defense in human airway epithelial cells. The success of overexpression of NOS2 in CF cells, or pretreatment with IFN, in protection from viral infection indicates that these approaches are promising in prevention of CF lung infection. Although less effective, provision of NO donors provided significant reduction of viral production and may be an alternative strategy for treatment of CF patients.

Experimental Procedures

Cell Culture, Virus, and Cytokines

HAEC were obtained through bronchoscopy brushing, from explanted lungs, or from segments of bronchus obtained from surgery and cultured by methods previously described (Guo et al., 2000; Uetani et al., 2000). An aliquot of cultured cells was immunostained to confirm epithelial phenotype. In addition, all cells were genotyped for 86 common *CFTR* mutations by Genzyme Genetics (Boston, Massachusetts). All eight samples from explant CF lungs were confirmed to be homozygous *ΔF508/ΔF508*. Eleven samples from control non-CF lungs were all wild-type *CFTR*.

A549 cells and CV-1 cells were maintained as previously described (Choudhary et al., 2001; Guo et al., 2000). HPIV3 (HA-1, NIH 47885) was a kind gift from Dr. De. Human IFN-γ was a gift from Genentech Inc. (South San Francisco, California). The IFN-α was purchased from Sigma-Aldrich (St. Louis, Missouri). Recombinant human IL-1β and TNF-α were from Genzyme.

RNA Isolation and Northern Analysis

Total RNA was extracted by GTC-CsCl gradient method (Erzurum et al., 1993a). Northern analysis was carried out using ^{32}P-dCTP-labeled human NOS2 cDNA by methods previously described (Uetani et al., 2000).

Plaque Assay and Immunofluorescent Staining

Culture supernatants were collected, and the yield of infectious HPIV3 in cells that underwent specific treatments was measured by plaque assay on CV-1 cells as previously described (De et al., 1995). 24 hr postinfection, cells cultured on cover slides were stained for HPIV3 by the method previously described (Choudhary et al., 2001).

^{35}S-Methionine Labeling and Immunoprecipitation

CF and NL cells in 12-well plate were infected with HPIV3 at 0.1 moi. At 12 hr postinfection, the medium was replaced with methionine-free DMEM and incubation was continued in 37°C. At 14 hr postinfection, the cells were labeled with 50 μCi of ^{35}S-methionine in 1 ml methionine-free DMEM for 6 hr. Cells were washed with DPBS and cell lysates were prepared and 20 μg of protein was immunoprecipitated by antibody against HPIV3 N-protein as previously described (De et al., 2000) and analyzed in an SDS-10% polyacrylamide gel.

IL-6 and IL-8 ELISA

Production of human IL-6 and IL-8 in the supernatant from CF and NL cells 24 hr after HPIV3 infection was evaluated using Quantikine human IL-6 and IL-8 ELISA (R&D Systems, Minneapolis, Minnesota). All samples were diluted ten times using appropriate calibration buffer.

Custom cDNA Microarray and Affymetrix Gene Array

RNA extracted from CF and NL cells at baseline or after 8 hr IFN treatment were evaluated for gene expression profile using custom-constructed cDNA microarray as previously described (Frevel et al., 2003). The ISG/AU/dsRNA array used in this study contains 1013 ISGs, 1464 AU-rich genes, 18 genes potentially involved in AU directed mRNA decay, 54 ribosomal genes, 288 dsRNA-responsive genes, and 84 housekeeping genes (NOS2 is not on this array).

Affymetrix HG-U133A GeneChips were also used in this study to evaluate baseline gene expression in CF and NL cells as previously described (Lipshutz et al., 1999; Yang et al., 2000).

Western Analysis

Whole-cell lysates were prepared and Western analysis performed as previously described (Uetani et al., 2000). The primary antibodies used included rabbit polyclonal antibody against NOS2 (Merck, Rahway, New Jersey), rabbit polyclonal antibody against C terminus of IRF-1 (Santa Cruz Biotechnology, Santa Cruz, California), rabbit polyclonal antibody against PKR (Carpick et al., 1997), mouse monoclonal antibody against RNase L (Dong and Silverman, 1995), rabbit polyclonal antibodies against MxA and HPIV3 N-protein (Choudhary et al., 2001), and rabbit polyclonal antibody against 2′, 5′ OAS1 (Ghosh et al., 2001).

WCE and EMSA

WCE were prepared and EMSA performed by methods previously described (Guo et al., 1997; Uetani et al., 2000). To specifically identify NF-κB, IRF-1, and STAT1α (p91) proteins in binding complexes, 2–4 μg of rabbit anti-p65, anti-p50, anti-IRF-1, or anti-STAT1α (p91) polyclonal Ab (Santa Cruz Biotechnology) was added to the binding reaction mix and incubated for 30 min at room temperature before adding the ^{32}P-labeled oligonucleotide.

NOS2 Expression Construct and Transient Transfection

Human NOS2 expression construct was made by inserting full-length NOS2 cDNA into a pAVS6 vector (Erzurum et al., 1993b). A control construct was also made by inserting reverse sequence NOS2 cDNA into a pAVS6 vector. Transient transfection was performed on cells at 90% confluence using LipofectAMINE PLUS reagent (Invitrogen Corporation, Carlsbad, California).

Nitrite and Nitrate Quantitation

NO production was quantitated by measuring total nitrite and nitrate in the media, using ISO-NO MarkII isolated nitric oxide meter and nitric oxide sensor (ISO-NOP) (World Precision Instruments, Inc., Sarasota, Florida). Data were collected and analyzed by Duo18.

Statistical Analysis

The data are reported as means ± standard deviation of the mean (SD). Two-tailed t test statistics or the Mann-Whitney test was used as appropriate at a significance level of 0.05.

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Temporal activation of NF-κB regulates an interferon-independent innate antiviral response against cytoplasmic RNA viruses

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NF-κB is known to exert its antiviral innate immune response via the IFN-β-induced Janus kinase/signal transducers and activators of transcription pathway. However, our current studies have demonstrated that activated NF-κB is capable of directly establishing an antiviral state independent of IFN or secreted soluble factor(s) against two highly pathogenic respiratory RNA viruses. Human parainfluenza virus type 3, a mildly cytopathic virus that induced NF-κB very early during infection was converted to a virulent virus after NF-κB inhibition. In contrast, a highly cytopathic virus, human respiratory syncytial virus that induced NF-κB late during infection, was converted to a mildly cytopathic virus after NF-κB induction before virus replication. This interconversion of cytopathic phenotypes of viruses after NF-κB modulation was further shown to be independent of IFN and soluble secreted factors(s). Moreover, tumor necrosis factor α (TNF-α) and IL-1β elicited an antiviral response, which was NF-κB-dependent. Thus, NF-κB induction directly confers an essential innate antiviral response against human parainfluenza virus type 3 and respiratory syncytial virus, which is independent of IFN-inducible factor(s).

Innate immune response initiated by the infected host cells constitutes the first line of defense against foreign pathogens including viruses, before orchestrating a well organized adaptive immune response. NF-κB, a family of evolutionarily conserved transcription factors, represents an important modulator of innate and adaptive immune function required for optimal host defense (1–4). Viruses have evolved to activate NF-κB, either by double-stranded RNA intermediate or activation of Toll-like receptors (TLRs), leading to nuclear translocation of NF-κB (5–7). In the nucleus, NF-κB binds to its cognate promoter sites to activate an array of genes, including proinflammatory cytokines, chemokines, and adhesion molecules (7). These molecules are involved in initiating adaptive immunity process by recruiting immune cells to the site of infection. Apart from the adaptive immune responders, NF-κB's innate immune function is mediated by the activation of IFN-β, an important antiviral cytokine (8, 9), through which paracrine action activates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) antiviral pathway (8, 9).

We have used two viruses, human parainfluenza virus type 3 (HPIV-3) and human respiratory syncytial virus (RSV), to study the role of NF-κB activation in conferring essential innate antiviral response in human epithelial cells. These cells facing the luminal side (e.g., intestine, lung, and airway) have direct contact with the exterior milieu and are, therefore, the initial target for majority of pathogens, including viruses (10). Both HPIV-3 and RSV, belonging to the paramyxoviridae family, are enveloped single-stranded RNA containing viruses of negative polarity that replicates in the cytoplasm (11). These viruses are important human respiratory tract pathogens, causing high morbidity among infants, children, and immunocompromised adults manifesting disease states including, pneumonia, croup, and brochiolitis (11). To date, no effective vaccine or antiviral therapy exists for either of these viruses. Therefore, elucidation of innate immune antiviral response elicited by these viruses holds significant potential for development of effective antiviral therapies against these viruses.

In this article, we report that NF-κB is capable of signaling an innate antiviral response that is independent of IFN and the well established JAK/STAT antiviral pathway. The importance of NF-κB-mediated innate response was further borne out by our observation that the temporal nature of NF-κB induction profile exhibited by RSV and HPIV-3 had direct bearing on their respective cytopathic phenotype and replication capability. Moreover, proinflammatory cytokines like tumor necrosis factor-α (TNF-α) and IL-1β exerted a potent antiviral action, which was directly dependent on the NF-κB innate antiviral pathway. The antiviral role of NF-κB against these cytoplasmic RNA viruses is discussed.

Materials and Methods

Cells and Viruses. A549, CV-1 cells, WT and IKKγ$^{-/-}$ mouse embryonic fibroblasts (MEFs), and human epithelial-like fibrosarcoma cells (WT and STAT-1$^{-/-}$ cells) were cultured as described (10, 12–15). HPIV-3, RSV, and vesicular stomatitis virus (VSV) adenoviruses (Ads) were propagated in CV-1, HepG2, BHK, and HEK cells, respectively, as described (10, 14–16).

Plaque Assay. Plaque assay was performed as described (15). To visualize the cytopathic effect, the same dilutions of medium supernatants were similarly added to CV-1 cells, and the plaques were viewed by phase contrast microscopy (×10 objective). The plaque assay data shown in the figures represents the mean number of plaque-forming units/ml from three independent experiments with similar results.

Virus Infection. A549 cells pretreated with used pyrollidine dithiocarbamate (PDTC) (Calbiochem; 50 μM) for 4 h or infected with the Ads [200 multiplicity of infection (mois)] for 16 h were infected with HPIV-3 (0.1 moi) or RSV (0.1 moi), either in the absence or presence of PDTC. After 36 h postinfection, the medium supernatants were prepared for plaque assay (15). The MEFs and fibrosarcoma cells were similarly infected with HPIV-3 and RSV (0.1 moi).

Electrophoretic Mobility-Shift Assay (EMSA) and Luciferase Assay. Nuclear extracts were prepared from HPIV-3 (3 mois), RSV (1 moi) -infected A549 cells as described (17). The nuclear extracts

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Abbreviations: HPIV-3, human parainfluenza virus type 3; RSV, human respiratory syncytial virus; VSV, vesicular stomatitis virus; TNF-α, tumor necrosis factor α; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MEF, mouse embryonic fibroblast; PDTC, pyrollidine dithiocarbamate; moi, multiplicity of infection; IκB-SR, IκB super repressor; Ad, adenovirus; L-NMMA, N^G-monomethyl-L-arginine; CM, conditioned medium; TLR, Toll-like receptor; DN-MyD88, dominant negative TLR adaptor protein MyD88; NF-κB-Luc, NF-κB-luciferase.

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(8 μg of protein) were either incubated with ^{32}P-labeled NF-κB oligonucleotide probe in the presence of preimmune/normal rabbit serum (NRS) or p65 antibody (Santa Cruz Biotechnology), or incubated with a 50× molar excess of unlabeled specific WT or mutant NF-κB oligonucleotide probe, and analyzed as described (16, 17). For luciferase assay, A549 cells were transiently transfected with plasmids containing the 2× NF-κB promoter fused to the firefly luciferase gene (16) and *Renilla* luciferase (for normalization of transfection efficiencies) by using lipofectin (GIBCO/BRL) as described (16). Sixteen hours posttransfection, cells were infected with either HPIV-3 or RSV (1 moi) and 8 h (for HPIV-3) or 20 h (for RSV) postinfection, the cell lysates were prepared and assayed for firefly lucifease expression as described (16). The luciferase activity was normalized to the *Renilla* luciferase activity (dual luciferase assay from Promega). NF-κB-luciferase- (NF-κB-Luc) transfected A549 cells were also infected with Ads [Ad-GFP, IκB super repressor (Ad-IκB-SR), and Ad-expressing dominant negative TLR adaptor protein MyD88 (Ad-DN-MyD88)] for 12 h, followed by either mock infection or infection with RSV or HPIV-3 (1 moi) for 18 and 8 h, respectively. Similarly NF-κB-Luc-transfected A549 cells were infected with either Ad-GFP or Ad-IκB-SR for 12 h, followed by TNF-α or IL-1β treatment (20 ng/ml) for 2 h. The lysates from these cells were assayed for luciferase activity as described above. The luciferase assay results shown in the figures represent the average of three independent experiments and the standard deviations are shown as error bars.

Treatment of HPIV-3-Infected A549 Cells with N^G-monomethyl-L-arginine (L-NMMA), Conditioned Medium (CM), or IFN-β. A549 cells pretreated with 5 mm or 25 mM L-NMMA (Oxis International, Portland, OR) for 4 h were infected with HPIV-3 (0.1 moi) for 36 h in the absence or presence of L-NMMA. To prepare the CM, A549 cells were either mock infected or infected with HPIV-3 (0.1 or 1 moi) for 24 h. The resulting medium supernatants were added to Centricon units (Centricon Plus-20, 300,000-kDa cutoff; Millipore) and centrifuged per manufacturer's direction. After centrifugation, the membrane flow-through supernatants were checked for the absence of virus by infecting fresh A549 cells. Once the absence of virus was confirmed, the mock or the HPIV-3 CMs were added to Ad-infected cells simultaneously during adsorption (2 h at 37°C) of HPIV-3 (0.1 moi). The CM was present during the course of the infection (36 h). IFN-β (2,000 units/ml) (PBL) was similarly added to Ad-infected cells simultaneously during adsorption (2 h at 37°C) of HPIV-3 (0.1 moi), and it was present during the course of the infection (36 h). The medium supernatants from L-NMMA-, CM-, or IFN-β-treated A549 cells infected with HPIV-3 were processed for plaque assay analysis.

RSV and VSV Infection of A549 or Human Fibrosarcoma Cells Pretreated with Either TNF-α or IL-1β in the Presence of Either Ad-GFP or Ad-IκB-SR. A549 or human fibrosarcoma cells were pretreated with TNF-α or IL-1β (20 ng/ml) (R & D Systems) for either 8 or 16 h. After pretreatment, the cells were infected with RSV, HPIV-3, or VSV (0.1 moi) for 36 h in the absence of these cytokines. A549 cells were also infected with Ads (Ad-GFP or Ad-IκB-SR) for 12 h, followed by TNF-α or IL-1β pretreatment (20 ng/ml) for 16 h. These cells were then infected with either RSV or VSV (0.1 moi) for 36 h in the absence of these cytokines. The medium supernatants from these cells were then subjected to plaque assay analysis on CV-1 cells.

RSV and VSV Infection of A549 or Human Fibrosarcoma Cells Pretreated with Either TNF-α or IL-1β CM in the Presence of Anti-TNF-α or Anti-IL-1β Neutralizing Antibodies, Respectively. CM obtained from untreated and TNF-α- or IL-1β-treated (20 ng/ml) A549 (8, 16, or 24 h) or human fibrosarcoma (24 h) cells were incubated with either control or respective cytokine-neutralizing antibodies (300 ng/ml; R & D Systems) overnight at 4°C. After incubation, the medium was added to fresh A549 or human fibrosarcoma cells for 16 h pretreatment before adding RSV or VSV (0.1 moi). The medium supernatants obtained from these cells after 36 h virus infection were subjected to plaque assay analysis on CV-1 cells

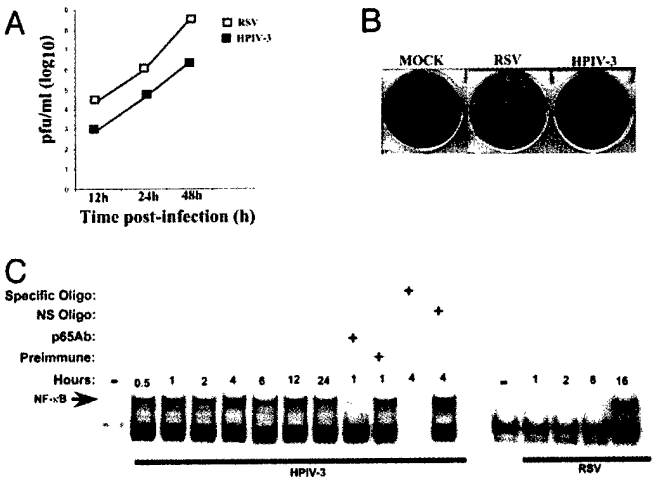

Fig. 1. Replication kinetics and NF-κB-induction profile of HPIV-3 and RSV in human lung epithelial A549 cells. (*A*) A single-step growth kinetics of HPIV-3 and RSV (0.1 moi) in A549 cells was determined by plaque assay analysis. (*B*) Cytopathic effect analysis (48 h postinfection) of HPIV-3 and RSV (0.1 moi) from infected A549 cells was determined after addition of same dilution of A549 medium supernatants to CV-1 cells for plaque assay analysis. (*C*) NF-κB EMSA using nuclear extracts from uninfected (−) and RSV-infected (1, 2, 6, and 16 h postinfection) A549 cells, and HPIV-3-infected (0.5–24 h postinfection) A549 cells in the absence or presence of NF-κB p65 antibody (Ab), preimmune serum, specific NF-κB unlabeled probe, or mutant NF-κB unlabeled probe (NS) as indicated.

Results

Temporal Activation of NF-κB by HPIV-3 and RSV in Human Lung Epithelial Cells. Human lung epithelial A549 cells were initially tested for susceptibility to HPIV-3 and RSV infections. We observed that HPIV-3 is significantly less cytopathic than RSV in these cells; the cells that are the primary target of these viruses during productive infection (ref. 11 and Fig. 1 *A* and *B*). A single-step growth kinetics of these viruses (0.1 moi) at 12, 24, and 48 h postinfection revealed that HPIV-3 titer was significantly lower (two logs) compared with RSV (Fig. 1*A*). These differences can also be directly visualized by their ability to form syncytia; cytopathic effect analysis (Fig. 1*B*) of these viruses at the same dilution of medium supernatants showed few plaques for HPIV-3, whereas RSV completely disseminated the cells. These results demonstrated that HPIV-3 replicated poorly in A549 cells, whereas RSV was highly cytopathic.

We next investigated whether these viruses possessing widely different cytopathic phenotype also differ in their induction of NF-κB, which is an important innate immune responder (1–4). EMSA (Fig. 1*C*) performed with nuclear extracts obtained from RSV- or HPIV-3-infected A549 cells, revealed rapid NF-κB DNA-binding activity very early after HPIV-3 infection (30 min postinfection) before initiating its full replicative cycle (12–16 h postinfection; refs. 18 and 19). In contrast, RSV-induced NF-κB DNA-binding activity was observed considerably later (16 h) after the onset of replication (10–12 h postinfection; ref. 20), similar to that reported (21, 22). Specificity of the HPIV-3-induced NF-κB complex formation was demonstrated by super-

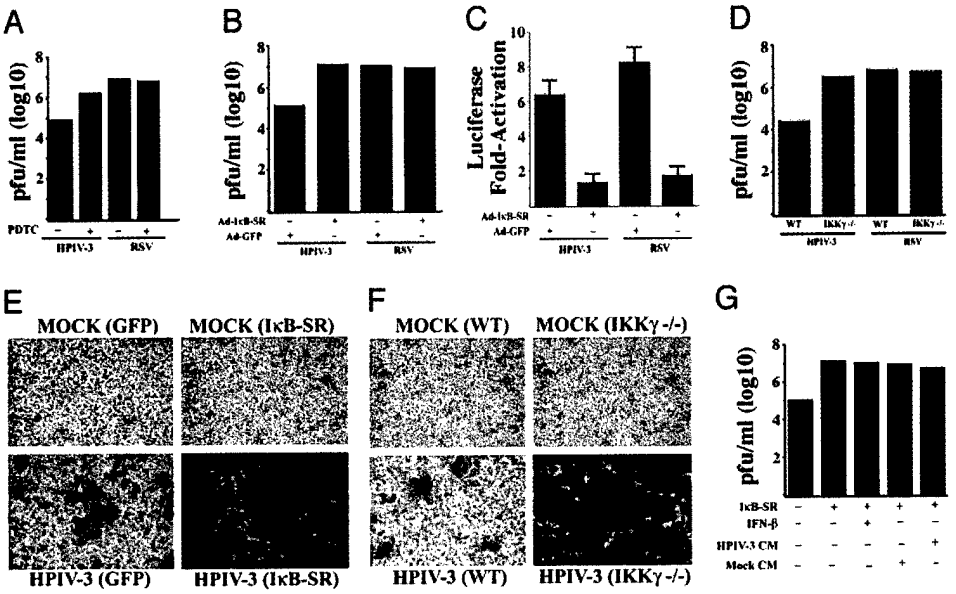

Fig. 2. Inhibition of NF-κB activation increases HPIV-3, but not RSV, replication and cytopathogenicity in an IFN- and/or soluble factor(s)-independent manner. Plaque assay analysis using medium supernatants from A549 cells mock infected or infected with either HPIV-3 or RSV in the absence or presence of PDTC (50 μM) (*A*), or A549 cells infected with HPIV-3 or RSV in the absence or presence of prior infection with Ads encoding the IκB-SR or GFP(*B*). (*C*) Expression of transfected NF-κB-Luc in A549 cells infected with HPIV-3 or RSV in the presence of IκB-SR or control GFP. (*D*) Plaque assay analysis using medium supernatants from IKKγ$^{-/-}$ or WT MEFs infected with either HPIV-3 or RSV. Phase contrast microscopic picture of CV-1 cells incubated with same dilutions of medium supernatants obtained from A549 cells infected with HPIV-3 and Ad-GFP or Ad-IκB-SR (*E*) and HPIV-3-infected WT or IKKγ$^{-/-}$ MEFs (*F*). (*G*) Plaque assay analysis using medium supernatants from A549 cells infected with HPIV-3 and Ad-GFP or Ad-IκB-SR and treated with IFN-β (2,000 units/ml). Similar analysis was performed with medium supernatants from A549 cells infected with HPIV-3 and Ad-GFP or Ad-IκB-SR in the presence or absence of either mock CM or HPIV-3 CM (cleared free of virus).

shift analysis with p65 antibody and competition with unlabeled WT, but not mutant oligonucleotide probe (Fig. 1*C*).

Inhibition of NF-κB Activation Results in Increased Replication and Cytopathogenicity of HPIV-3 Independent of IFN. The differential kinetics of NF-κB induction displayed by RSV and HPIV-3 raised the possibility that these viruses may have evolved to regulate the activation of NF-κB to strategically manipulate the antiviral defense mechanism exerted by the host for efficient replication, leading to higher cytopathic phenotype. To examine this possibility, we monitored the replication capability of HPIV-3 and RSV in cells where NF-κB was inactivated. By using PDTC, a general NF-κB inhibitor (ref. 23 and Fig. 2*A*), during infection resulted in a significant (30-fold) increase in HPIV-3 titer. In contrast, PDTC had no effect on RSV titer (Fig. 2*A*). These results were further confirmed by expressing the Ad-IκB-SR (32A/36A) (16, 24) in HPIV-3-infected cells. As shown in Fig. 2*B*, IκB-SR expression lead to dramatic increase (100-fold) in HPIV-3 titer, whereas RSV titer remained unchanged. The Ad-IκB-SR used in these studies was functional, because Ad-IκB-SR, but not control Ad-GFP, inhibited NF-κB-Luc induction by HPIV-3 and RSV in A549 cells (Fig. 2*C*). Finally, virus obtained from infected WT and IKKγ$^{-/-}$ MEFs (12) demonstrated dramatic augmentation (100-fold) of HPIV-3, but not RSV replication in IKKγ$^{-/-}$ cells (Fig. 2*D*). The significant increase in HPIV-3 cytopathogenicity and viral titer could be clearly visualized by increased syncytia formation after NF-κB inactivation (Fig. 2 *E* and *F*). The observed conversion of mildly cytopathic HPIV-3 into a virulent form, similar to RSV, after inhibition of NF-κB, suggested an important role of NF-κB in antiviral defense. These results indicate that rapid activation of NF-κB before HPIV-3 replication constitutes an essential antiviral host defense in human lung epithelial cells, as well as in mouse fibroblasts.

We next examined whether the conversion of a mildly cytopathic virus, HPIV-3, to a virulent one on NF-κB inhibition was due to the lack of IFN-β [a gene which is stimulated in HPIV-3-infected epithelial cells (25, 26)] or soluble antiviral secreted factor(s) production. As shown in Fig. 2*G*, addition of exogenous IFN-β (2,000 units/ml) to the NF-κB-inactivated cells at the time of HPIV-3 infection failed to inhibit the increased infectivity and cytopathogenicity of HPIV-3. Similar results were obtained after addition of IFN-α (data not shown). The potential involvement of putative secretory antiviral soluble factor(s) were also eliminated, because CM supernatant obtained from HPIV-3-infected A549 cells (cleared free of virus) or mock-infected cells added to Ad-IκB-SR- and HPIV-3-infected cells at the time of HPIV-3 infection, failed to repress the increased virus infectivity (Fig. 2*G*). Thus, it seems that NF-κB-mediated anti-HPIV-3 activity is conferred by establishing an intracellular antiviral state independent of IFN and/or soluble factor(s).

Inhibition of RSV Replication After Specific Induction of NF-κB by TNF-α or IL-1β. If indeed NF-κB is an essential innate antiviral mediator as shown above, we hypothesized that RSV may have maintained its highly cytopathic phenotype as a result of deregulating NF-κB activation by inducing it late in the replication cycle. If such induction constitutes the mechanism used by RSV to evade host's NF-κB-dependent antiviral response for preservation of its high virulence, activation of NF-κB before the replication of RSV may confer an antiviral state. To examine this possibility, we pretreated A549 cells with TNF-α (27) or IL-1β (28), which are potent inducers of NF-κB, before RSV infection. Pretreatment of cells with TNF-α or IL-1β (Fig. 3*A*) severely restricted RSV replication with a decrease in virus titer by 1,000-fold. However, treatment of A549 cells with TNF-α or IL-1β 10–12 h postinfection (after replication initiation) failed to restrict RSV replication (data not shown). It is important to note that in these studies, TNF-α and IL-1β were present only

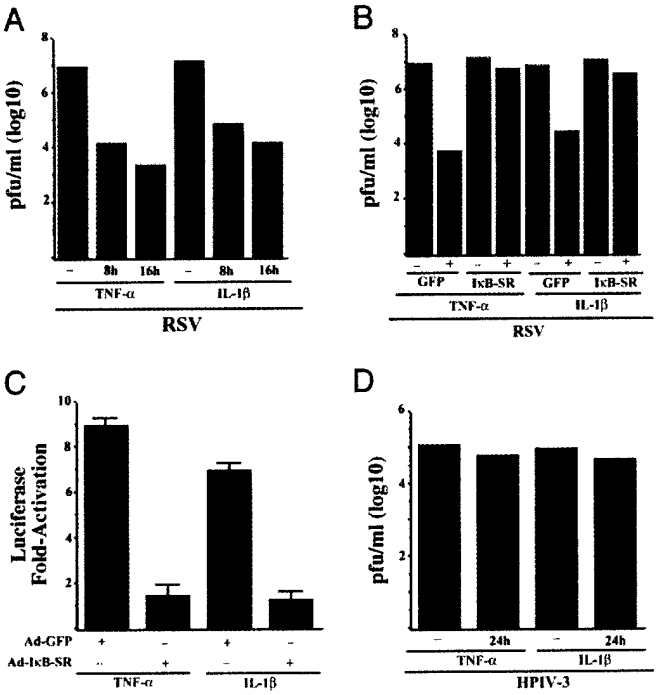

Fig. 3. Effect of TNF-α and IL-1β pretreatment on NF-κB-dependent restriction of RSV replication and cytopathogenicity. (*A*) Plaque assay analysis of medium supernatants from A549 cells pretreated with 20 ng/ml TNF-α or IL-1β for 8–16 h before RSV infection. (*B*) Plaque assay analysis of culture supernatants from IκB-SR or GFP expressing A549 cells, pretreated with either TNF-α or IL-1β for 16 h before RSV infection. (*C*) Expression of transfected NF-κB-Luc in A549 cells treated with either TNF-α or IL-1β (20 ng/ml for 2 h) in the presence of IκB-SR or control GFP. (*D*) Plaque assay analysis of medium supernatants from A549 cells pretreated with 20 ng/ml TNF-α or IL-1β for 24 h before HPIV-3 infection.

during the pretreatment period, but not during virus infection. These results strongly suggest that establishment of NF-κB-dependent antiviral state before infection was sufficient to restrict the replication of RSV.

Because TNF-α and IL-1β induces additional signaling pathways (e.g., c-Jun N-terminal kinase and extracellular signal-regulated kinase) apart from NF-κB (29, 30), we investigated whether the antiviral action of TNF-α and IL-1β is specifically mediated by activated NF-κB. Inhibition of NF-κB activation after expression of IκB-SR significantly reverted the antiviral action of TNF-α and IL-1β (Fig. 3*B*) toward RSV. In control experiments, TNF-α or IL-1β treatment of A549 cells transfected with NF-κB-Luc led to significant induction of NF-κB activity, which was inhibited when the cells were infected with Ad-IκB-SR (Fig. 3*C*). These results demonstrated the potent antiviral action of TNF-α and IL-1β is indeed mediated specifically via the NF-κB signaling pathway, and the latter pathway plays an important innate antiviral role in host cells, only when it is activated before virus replication. Interestingly, TNF-α or IL-1β (Fig. 3*D*) pretreatment failed to exert an anti-HPIV-3 activity. Presumably, HPIV-3-mediated induction of NF-κB during the normal course of infection represents the optimal threshold value for its antiviral activity, which is not augmented further by TNF-α or IL-1β treatment.

NF-κB-Dependent Antiviral Response by TNF-α and IL-1β Is Mediated Independent of IFN and Soluble Secreted Factor(s). Next, we investigated whether IFN and/or soluble factor(s) are involved in eliciting the NF-κB-dependent antiviral mechanism of TNF-α and IL-β against RSV. As shown in Fig. 4 *A* and *B*, the medium

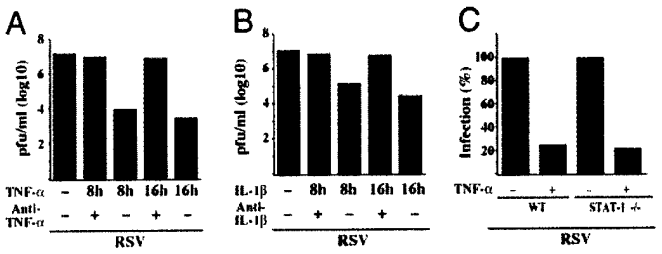

Fig. 4. The noninvolvement of IFN and/or soluble secretory factor(s) in mediating the NF-κB-dependent antiviral response elicited by TNF-α and IL-1β. Plaque assay analysis of medium supernatants from A549 cells infected with RSV after a 16-h pretreatment of A549 cells with medium obtained from TNF-α- (*A*) or IL-1β- (*B*) treated (8 or 16 h) A549 cells in the presence of control antibody or anti-TNF-α (*A*) or anti-IL-1β (*B*) neutralizing antibodies. (*C*) Plaque assay analysis using medium supernatants from WT or STAT-1$^{-/-}$ cells untreated or pretreated with TNF-α (20 ng/ml for 16 h) before infection with RSV. The percent infection indicated was calculated based on a ratio of number of plaques obtained in the presence of TNF-α over the number obtained from untreated cells.

supernatant obtained from TNF-α- (Fig. 4*A*) or IL-1β- (Fig. 4*B*) treated A549 cells (cytokine CM) when treated with anti-TNF-α or anti-IL-1β neutralizing antibodies, respectively, and added to fresh A549 cells for pretreatment before virus infection, failed to inhibit RSV replication. The specificity of these cytokine-neutralizing antibodies was borne out by the observation that they failed to inhibit the antiviral action of IFN and the TNF-α and IL-1β CM retained its antiviral property even in the presence of anti-IFN neutralizing antibody (data not shown).

The noninvolvement of soluble secreted factor(s), including IFN, in exerting the NF-κB-dependent antiviral state was further shown by using WT 2fTGH- and IFN-insensitive STAT-1 null (STAT-1$^{-/-}$) U3A human epithelial-like fibrosarcoma cells (31). The lack of JAK/STAT signaling pathway was shown to have no effect on NF-κB signaling cascade induced by TNF-α treatment (32). Similar to A549 cells, TNF-α (Fig. 4*C*) or IL-1β (data not shown) pretreatment established an antiviral state for RSV in both WT and STAT-1$^{-/-}$ cells. Moreover, additional soluble secreted factor(s) were not involved during establishment of the antiviral state in WT or STAT-1$^{-/-}$ cells, as tested by performing similar experiments described in Fig. 4 *A* and *B* (data not shown). These results suggested that IFN and/or soluble secreted factor(s) are not involved in exerting a NF-κB-dependent antiviral state.

Use of MyD88 by HPIV-3 for NF-κB Induction. Because our results have suggested that a critical time frame of NF-κB activation in infected cells dictates the antiviral function of NF-κB, we investigated the mechanism(s) that may be involved in conferring the difference in postinfection NF-κB induction profile exhibited by HPIV-3 (rapid induction) and RSV (late induction). Recently TLRs have been shown to be used by RNA cytoplasmic viruses for rapid activation of NF-κB in infected cells (5, 6, 8). Because MyD88 is an essential TLR adaptor protein required for optimal TLR-dependent NF-κB activation (33, 34), we investigated the requirement of MyD88 in transducing RSV- and HPIV-3-mediated NF-κB activation signal.

Whereas infection of A549 cells with Ad-DN-MyD88 abrogated NF-κB-Luc activation by HPIV-3, Ad-DN-MyD88 failed to inhibit NF-κB induction by RSV (Fig. 5*A*). Moreover, the requirement of MyD88 in HPIV-3-mediated induction of NF-κB was borne out by the observation that expression of DN-MyD88 resulted in drastic increase in HPIV-3 replication and cytopathogenicity (Fig. 5 *B* and *C*), which was similar to that observed after expression of IκB-SR (Fig. 2 *B* and *E*). These results indicate that in human lung epithelial cells, HPIV-3 and RSV uses two

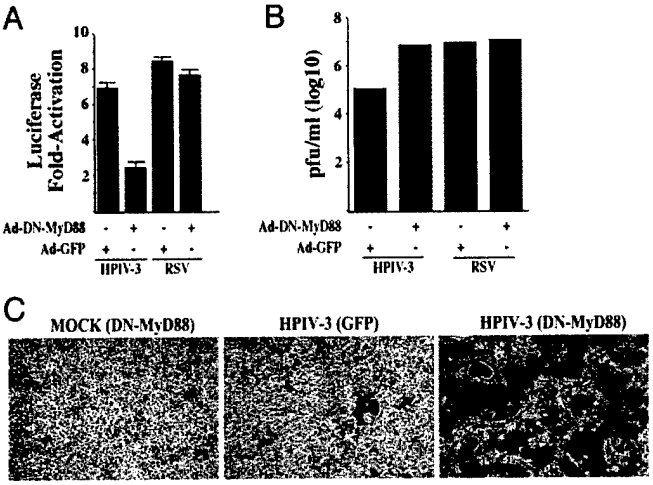

Fig. 5. Differential requirement of MyD88 for NF-κB induction by HPIV-3 and RSV. (*A*) Expression of transfected NF-κB-Luc in A549 cells infected with HPIV-3 or RSV in the presence of DN-MyD88 or control GFP. (*B*) The indicated medium supernatants from A549 cells infected with HPIV-3 or RSV in the absence or presence of prior infection with Ads encoding the DN-MyD88 or GFP were subjected to plaque assay analysis. (*C*) Phase contrast microscopic picture of CV-1 cells incubated with same dilutions of medium supernatants obtained from A549 cells infected with HPIV-3 and Ad-GFP or Ad-DN-MyD88.

alternative mechanisms, MyD88-dependent and -independent pathways, respectively, to induce NF-κB. Moreover, MyD88-dependent and -independent pathways adopted by these two viruses to either induce NF-κB rapidly (HPIV-3) or late after replication initiation (RSV), respectively, may have a direct bearing on their respective cytopathogenic phenotype.

Discussion

In this article, we have established that two medically important human respiratory tract pathogens, HPIV-3 and RSV, respond differentially to innate response elicited by NF-κB. The mildly cytopathic virus, HPIV-3 (which induces NF-κB early during infection), was converted to a virulent virus as a result of increased replication after inhibition of rapid NF-κB induction by HPIV-3. In contrast, the replication of RSV (which induces NF-κB late in infection) was not altered in NF-κB-inhibited cells. However, specific induction of NF-κB by TNF-α or IL-1β before virus replication rendered an antiviral state against RSV, converting this highly cytopathic virus to a less virulent virus. Similar results with drastic inhibition of viral replication after induction of NF-κB before virus infection were obtained when another highly cytopathic RNA cytoplasmic virus, VSV, which possesses similar high titer like RSV in A549 cells (data not shown), and fails to induce NF-κB in these cells (21) was allowed to infect TNF-α- and IL-1β-pretreated cells (see Fig. 7, which is published as supporting information on the PNAS web site, www.pnas.org). Moreover, similar to RSV, anti-VSV activity of TNF-α and IL-1β was elicited specifically by NF-κB, which is independent of IFN and soluble secreted factors (see Fig. 8, which is published as supporting information on the PNAS web site). These results demonstrated that NF-κB is also capable of conferring an essential IFN-independent antiviral activity against a virus that does not induce its activity during natural infection.

It is interesting to note that although the antiviral potential of TNF-α has been reported earlier (35, 36), the mechanism(s) involved in conferring the antiviral response was not known. In our current study, we have demonstrated that the antiviral activity of TNF-α is mediated directly by NF-κB, which is independent of IFN. In addition, we have demonstrated the ability of IL-1β to act as a potent antiviral cytokine, which exerts its IFN-independent antiviral activity by inducing NF-κB. The ability of two NF-κB-inducing cytokines to severely restrict virus replication similar to IFN demonstrated the importance of NF-κB in antiviral defense. Moreover, the noninvolvement of IFN in exerting a NF-κB-dependent antiviral response was borne out by previous reports that infection of STAT-$1^{-/-}$ cells yielded similar HPIV-3 titer compared with the WT cells (13), and RSV is insensitive to the antiviral action of IFN-α/β in A549 cells (37). Our results demonstrating that TNF-α and IL-1β exert their NF-κB-dependent antiviral action independent of IFN were recently validated, because microarray analysis did not reveal induction of IFN-α/β genes after treatment of cells with either TNF-α or IL-1β (38). In addition, nitric oxide (NO) production after inducible nitric oxide synthase (iNOS) induction through NF-κB (39) was not the antiviral factor, because A549 cells treated with the iNOS-competitive inhibitor L-NMMA during HPIV-3 infection resulted in no significant differences in HPIV-3 virus titer (data not shown). Moreover, the NF-κB-dependent antiviral action is not mediated by IFN-γ, because the nonimmune cells used in our studies are incapable of inducing IFN-γ gene.

Because NF-κB-mediated antiviral response critically relies on the time frame of its activation after virus infection, we further demonstrated that at least for RSV and HPIV-3, the temporal nature of NF-κB induction appears to depend on the use of MyD88. (33, 34). HPIV-3 used the MyD88-dependent pathway to rapidly induce NF-κB, probably after interaction of HPIV-3 envelope protein(s) with cell-surface TLRs during virus entry. In support of rapid activation of NF-κB by HPIV-3, a previous study (26) has reported the induction of MHC-I (a gene whose expression is regulated by transactivating function of NF-κB) by UV-irradiated (replication incompetent) HPIV-3 and UV-inactivated HPIV-3-induced NF-κB in A549 cells (data not shown). Further studies will be needed to identify the specific TLRs involved in NF-κB activation by HPIV-3. In contrast to HPIV-3, RSV induced NF-κB late after replication initiation via the MyD88-independent pathway. In that context, previous studies have shown that in contrast to alveolar macrophages or monocytes, RSV activated NF-κB late during infection in lung

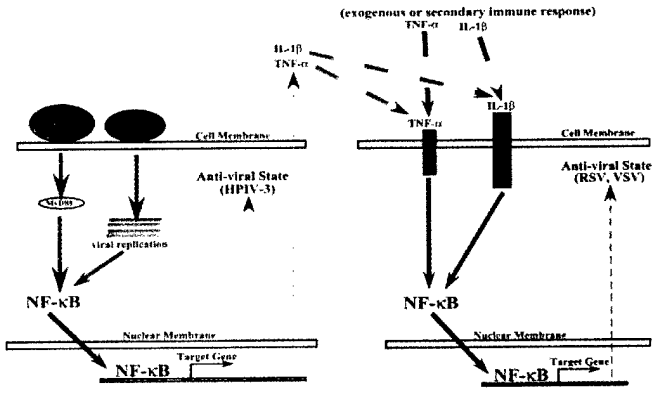

Fig. 6. A model depicting NF-κB-mediated innate antiviral response independent of IFN. Rapid activation of NF-κB by viruses like HPIV-3 via the MyD88 pathway early during infection in a replication-independent manner confers an intracellular antiviral state in the infected cells (*Left*). Similarly, viruses like RSV that induce NF-κB late after infection in a replication-dependent manner, produce TNF-α and/or IL-1β, which, by means of the paracrine mechanism, may prime uninfected cells (*Right*) after binding to its cognate receptors to establish an NF-κB-dependent antiviral state. In addition, exogenously added TNF-α or IL-1β, and the production of these cytokines after secondary adaptive response by immune cells, could prime uninfected cells to activate NF-κB-mediated antiviral response against viruses like VSV that do not induce NF-κB. TNFR, TNF-α receptor; IL-1βR, IL-1β receptor.

epithelial cells (21, 22). Moreover, the NF-κB activation by RSV in these cells were replication dependent, because UV-inactivated RSV (data not shown and ref. 40), and virus replication inhibitors (22) failed to activate RSV-dependent NF-κB induction in A549 cells. Similar to our observation, a recent study (41) has also reported TLR4-independent and replication-dependent activation of NF-κB by RSV in lung epithelial cells. In addition to MyD88, we observed a differential requirement of phosphatidylinositol 3-kinase (PI3K) (42) for induction of NF-κB by HPIV-3 and RSV. Whereas RSV failed to induce NF-κB in A549 cells after inhibition of PI3K activity (ref. 43 and data not shown), such inhibition had no effect on HPIV-3-mediated NF-κB activation (data not shown).

Based on our results, we propose a model for the direct establishment of an IFN-independent innate antiviral state after NF-κB activation (Fig. 6). Infection of human epithelial cells with HPIV-3 rapidly induces (replication independent) NF-κB via the MyD88-dependent IKK/IκB pathway, leading to the establishment of an antiviral state. HPIV-3, a mildly cytopathic, TNF-α-nonproducing virus (25, 26), thus induces the NF-κB antiviral pathway rapidly to restrict its own replication in infected cells. In contrast, NF-κB failed to exert its antiviral function against viruses, like RSV, which activated NF-κB via a MyD88-independent and replication-dependent pathway. Although RSV and VSV circumvents the antiviral activity of NF-κB in infected cells, activation of NF-κB by TNF-α and IL-1β (proinflammatory cytokines whose gene is regulated by transactivating function of NF-κB) before virus replication established an intracellular antiviral state. Thus, in the absence of IFN sensitivity (37) and evasion of NF-κB-dependent antiviral response, TNF-α and/or IL-1β produced by RSV-infected cells (44) may prime uninfected cells by means of binding to their cognate receptors, to restrict the spread of RSV by activating NF-κB. Moreover, TNF-α and/or IL-1β produced after secondary adaptive response by immune cells (45, 46), could prime uninfected cells to create an NF-κB-dependent antiviral state against viruses such as VSV, that do not induce NF-κB (21), and fail to produce IFN from infected cells (47).

In conclusion, we report an innate antiviral immune response that is independent of the well established IFN-induced JAK/STAT pathway, and demonstrate that this innate antiviral response is directly mediated by NF-κB after its activation, either by a virus or by proinflammatory cytokines like TNF-α and IL-1β. Thus, NF-κB acts as an essential host antivirulence factor to restrict the systemic spread of pathogens by not only producing IFN-β but also by being capable of directly establishing an IFN-independent intracellular antiviral state against several RNA cytoplasmic viruses by an alternative pathway.

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